Multilocus Genotyping of Giardial Assemblages Associated with Diarrhea in Children and Adults in South India

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CERTIFICATE

This is to certify that the thesis entitled **"Multilocus Genotyping of Giardial Assemblages Associated with Diarrhea in Children and Adults in South India"** is a bonafide work done by Dr. Shakti Laishram in partial fulfillment of the requirements for M.D. Branch IV (Microbiology) examination of the Tamil Nadu Dr. M.G.R. Medical University, Chennai to be held in April 2011. Her work was carried out under the guidance of Dr. Gagandeep Kang, Professor of Microbiology.

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CONTENTS

INTRODUCTION

Giardiasis, caused by *Giardia intestinalis* in humans manifests as a wide clinical spectrum ranging from asymptomatic infections to acute or chronic diarrhea as well as functional bowel disease (Mandell, Bennett et al. 2005). It is prevalent in developed, developing and underdeveloped countries. In India, prevalence rates reported in patients with diarrhea range from 0.4 to 70% (Sircar, Deb et al. 1984; Ramesh, Malla et al. 1991). Asymptomatic cyst passage has also been found to be as high as 50% (Kang, Mathew et al. 1998). Apart from diarrhea, giardial infection in children can result in long term faltering of growth, stunting and decreased cognitive function (Berkman, Lescano et al. 2002; Al-Mekhlafi, Azlin et al. 2005; Nematian, Gholamrezanezhad et al. 2008).

The species *Giardia intestinalis* consists of a highly heterogeneous group of organisms and is referred to as a species complex divided into 7 ‘assemblages’ or genotypes (A to G) with different host specificities. Of these, human infections are mainly caused by assemblage A and B. The remaining assemblages infect animals, though occasionally some have been found in humans, suggesting a zoonotic mode of transmission (Caccio, Beck et al. 2008; Foronda, Bargues et al. 2008). Different assemblages have been associated with increased risk of symptoms in different geographical regions, hence genotyping of giardial isolates is essential to understand the epidemiology and transmission ecology of the disease in a given setting or area. With the development of molecular tools, the organism has not only been genotyped to the assemblage level but also to ‘sub-genotypes’. The molecular methods used commonly include restriction fragment analysis and sequencing, using fast evolving markers like tpi, gdh and β-

giardin as well as conserved markers like SSU rRNA and ef1 (Monis, Andrews et al. 1999). Heterogeneity between the loci for both human and animal isolates has been reported, so a multilocus genotyping (MLG) approach is recommended to assign assemblage type (Caccio, Beck et al. 2008).

Keeping this in mind, this study aimed to characterize giardial isolates from south India to determine the predominant circulating assemblage using a *MLG approach* targeting the 3 most commonly used loci, tpi, gdh and β-giardin. This was carried out in children with and without diarrhea to detect any association of symptoms with a particular assemblage as well as adults with gastrointestinal symptoms. The usefulness of the 3 loci and any discordance between the loci was compared. Information on the circulating assemblages and association with symptoms can help plan future interventional measures to prevent giardiasis in this population.

AIMS AND OBJECTIVES

Aim

- To describe the assemblages of *Giardia intestinalis* in children and adults in South India using multiple genetic loci

Objectives of the study

1. To identify the common giardial assemblages associated with diarrhea in children and adults using multiple genetic loci
2. To co-relate severity of diarrhea and clinical presentation with giardial assemblages
3. To compare the 3 target genes used for detection of giardial assemblages
4. To detect the presence of any zoonotic assemblages in humans

REVIEW OF LITERATURE

History

The first description of the intestinal parasite *Giardia* was by Leewenhoek in 1681 who observed the organism in his own diarrheal stool (Adam 2001). In 1859, Lambl gave the first formal description of the parasite naming it *Cercomonas intestinalis*. This was followed by descriptions by Grassi in 1879 (*Dimorphus muris* later known as *Giardia muris* in rodents) and Kunstler in 1882 (provided the genus name *Giardia*). Starting in the late 1880s, the name of the organism underwent many changes based on morphology and host of origin. Blanchard suggested the name *Lamblia intestinalis* in honor of Lambl (1888), Stiles changed this to *Giardia duodenalis* (1902), Alexeieff synonymised the genus names *Lamblia* and *Giardia* (1914) and *Giardia lamblia* and *Giardia enterica* were later proposed by Kofoid and Christiansen (1915 and 1920 respectively). Based on morphological differences *Giardia lamblia* was distinguished from *Giardia muris* by Simon (1922). According to differences in morphology, Felice, in 1952, described the species names *Giardia duodenalis*, *Giardia muris* and *Giardia agilis*. Since then, the species names *Giardia duodenalis*, *Giardia intestinalis* and *Giardia lamblia* have been used interchangeably to describe the *Giardia* species infecting humans.

Taxonomy

Giardia is a microaerophilic, flagellate protozoan parasite and along with *Entamoeba* was long considered to be a pre-mitochondriate eukaryote. However, with the discovery of structures common to higher eukaryotes, specifically, splicoeosomal introns, ras-family of signal-transduction proteins, ATP-binding cassette drug transporters and Golgi apparatus, they are now

thought to be an example of eukaryotic diversity that have undergone reductive evolution (Samuelson 2002; Monis, Caccio et al. 2008). *Giardia* belongs to the order Diplomonadida and the family Hexamitidae. Felice, in 1952, introduced the classification of *Giardia* according to differences in morphology under the light microscope and the host of origin (Adam 2001; Monis, Caccio et al. 2008). By this classification, *Giardia duodenalis* trophozoites are pear shaped with a claw-shaped median body and infect human and other mammals; *Giardia agilis* trophozoites are long and slender with a tear drop-shaped median body, and infect amphibians; whereas *Giardia muris* trophozoites are shorter and rounder with a small rounded median body and infect rodents (Adam 2001). Morphological differences discernable under the electron microscope along with differences in host of origin lead to the description 3 more species – *G.ardeae,* which infects herons, *G.psittaci,* which infects psittacine birds and *G.microti,* which infects voles and muskrats (Erlandsen and Bemrick 1987; Feely 1988; Erlandsen, Bemrick et al. 1990).

Giardia duodenalis – the parasite and life cycle

Giardia duodenalis (henceforth referred to as *Giardia*) has five chromosomes and a genome size of approximately 11.7 Mb, with a GC content of 49% (Morrison, McArthur et al. 2007). The genome is compact with few introns and short mean intergenic distance compared to other protozoa (Morrison, McArthur et al. 2007). The organism has a limited repertoire of metabolic functions with no enzyme homologues for the Kreb's cycle, no genes for purine and pyrimidine biosynthesis and a limited capacity for amino acid metabolism (Morrison, McArthur et al. 2007). These limitations are overcome by scavenging for essential metabolic products from the host, thus pointing to genomic reduction as an adaptation to a parasitism. Although presumed

to be asexual, a recent study found that *Giardia* has low levels of allelic heterozygosity, probably by fusion of nuclei in the cyst stage followed by somatic homologous recombination (Poxleitner, Carpenter et al. 2008).

Giardia exists in two forms – trophozoite and cyst. The trophozoite (Figure 1) is pear shaped and 10-20 μm by 5-15 μm in size. It is binucleate with anteriorly placed identical nuclei and four pairs of flagella – two pairs laterally, one caudal and one ventral. The two nuclei have complete copies of the genome, are functionally equivalent and are both transcriptionally active (Yu, Birky et al. 2002). The flagellae arise from a basal body which lie in the cellular midline and have a 9+2 microtubule arrangement seen in most eukaryotes (Gillin, Reiner et al. 1996). The median body is a structure unique to *Giardia* spp. and is present in 50% of the trophozoites. The cytosol contains lysosomal vacuoles, ribosomes and glycogen granules, and in encysting trophozoites, golgi complexes are present (Adam 2001). On the ventral side of the body lies a sucking disc which helps in attachment of the trophozoite to the intestinal wall. The disc is concave in cross-section and ultra-structurally consists of repeating single layers of microtubules and has a striated appearance due to microribbons which wrap around the microtubule and tether it to the cytoplasm (Gillin, Reiner et al. 1996). In a fresh wet preparation, trophozoites exhibit 'falling leaf motility'. In swimming trophozoites, anterior, lateral and ventral flagella beat in the plane of the adherence disc, while the tail along with the caudal flagella beat in a direction perpendicular to the adherence disc. The trophozoites move along the plane of the adherence disc and change in direction is associated with a rudder like movement of the tail (Ghosh, Frisardi et al. 2001).

Fig 1 – *Giardia* Trophozoite and Cyst Stages

A) B) C)

A) Trichrome stained Trophozoite; B and C) Iodine Wet Mounts of Trophozoite and Cyst Images taken at 1000X; From http://dpd.cdc.gov

The cyst (Figure 1) is oval, measures 11-14 μm by 7-10 μm, has a thick outer shell, central axostyle and four nuclei and is the infective form of the parasite. After ingestion of cysts, excystation occurs on exposure to low pH in the stomach releasing a transient stage that divides into 4 trophozoites (Bernander, Palm et al. 2001). The trophozoites localize in the upper small intestine, especially the duodenum where they undergo longitudinal binary fission and multiply. In a study done using episomal transfection of a single nucleus and detection of the transfected episome by FISH, the trophozoite was seen to divide along the longitudinal axis with mirror-image symmetry at the plane of the adherence disc such that the left nucleus of the mother cell becomes the right nucleus of the daughter cell and vice-versa (Ghosh, Frisardi et al. 2001). The eight flagella of the parent cell are distributed in a semi-conservative manner such that each progeny receives a different set of four flagella from the parent cell and daughter cells than assemble a new set of complimentary flagella requiring three cell cycles to mature (Nohynkova, Tumova et al. 2006). Encystation occurs when the parasite passes down the intestinal tract on exposure to bile salts. The infective cyst is then passed in feces.

Epidemiology

Giardiasis is seen both in the developed and the developing world and is the most frequent intestinal protozoan infection with estimated 280 million cases occurring worldwide annually (Lane and Lloyd 2002). In resource poor countries, this parasite alone accounts for 2.5

million cases of diarrhea associated deaths and morbidity (Younas, Shah et al. 2008). In these countries, *Giardia* is acquired early during infancy and prevalence peaks at 15 to 30% in children younger than 10 years of age (Mandell, Bennett et al. 2005). Given its high prevalence among poor and underdeveloped communities, giardiasis along with cryptosporidiosis was included in the WHO 'Neglected Diseases Initiative' in 2004 (Savioli, Smith et al. 2006).

In India, giardiasis has been found to be prevalent throughout the country as demonstrated by several epidemiological studies (Table 1). In community based studies from North India, prevalence ranged from 5.5 to 70% (Walia, Ganguly et al. 1986; Ramesh, Malla et al. 1991; Bansal, Sehgal et al. 2004; Khurana, Aggarwal et al. 2005) with the highest reported prevalence (69.5%) in a low socio-economic group in Chandigarh (Ramesh, Malla et al. 1991). Two studies on pre-school children in Lucknow and Punjab found that around 30% of children were asymptomatic cyst passers (Walia, Ganguly et al. 1986; Awasthi and Pandey 1997). In studies among children with diarrhea the prevalence of giardiasis ranged from 4 to 20% (Mahendraker, Dutta et al. 1991; Thapa 1994; Jindal, Arora et al. 1995; Bhandari, Bahl et al. 1999; Kaur, Rawat et al. 2002). In studies from south India the prevalence of symptomatic disease in children and adults was 8-10% and 37.1% respectively (Shetty, Narasimha et al. 1990; Shenoy, Urs et al. 1998) while asymptomatic cyst passage varied from 2.45 to 53.8% (Subbannayya, Babu et al. 1989; Kang, Mathew et al. 1998). In a study carried out among asymptomatic school children in and around Chennai, the prevalence in rural areas was 16% while that in an urban setting was 22.6% (Fernandez, Verghese et al. 2002). In reports from Calcutta, *Giardia* as a cause of diarrhea accounted for 0.4 to 13.3% of cases (Sircar, Deb et al. 1984; Chatterjee, Thawani et al. 1989; Mukherjee, Chowdhury et al. 2009; Nair, Ramamurthy et

al. 2010), while a study on the general population in the north eastern state of Sikkim showed a prevalence of 5.9% (Mitra 1970). In a study conducted among food handlers, *Giardia* was the commonest parasite (Khurana, Taneja et al. 2008). This wide variation in the prevalence of *Giardia* may reflect its geographical distribution in the country or may also be due to different methodologies applied including the number of sequential stool samples tested per patient. As evidenced from the data, several studies in asymptomatic children and adults also found a high prevalence of giardiasis raising the question of whether these asymptomatic infections in addition to acting as a reservoir, themselves affect the nutritional status of the host.

As with any infectious disease, the impact of HIV on giardiasis has been studied and although not considered a classical opportunistic infection, the prevalence has been found to be higher among AIDS patients and is frequently associated with diarrhea (Angarano, Maggi et al. 1997; Punpoowong, Viriyavejakul et al. 1998; Moolasart 1999; Silva, Ferreira et al. 2005). In India, the prevalence of giardiasis in HIV infected patients ranged from 1.6 to >30% (Mohandas, Sehgal et al. 2002; Dwivedi, Prasad et al. 2007; Vignesh, Balakrishnan et al. 2007; Gautam, Bhalla et al. 2009), with some studies showing a higher prevalence in patients with diarrhea (Joshi, Chowdhary et al. 2002; Mohandas, Sehgal et al. 2002; Dwivedi, Prasad et al. 2007) and lower CD4 counts (Dwivedi, Prasad et al. 2007; Gautam, Bhalla et al. 2009).

Table 1: Studies on the Epidemiology of Giardiasis in India

Site of study	n	Population	%	Reference
North India				
Amritsar	150	Children < 3 yrs, chronic diarrhea	4	(Jindal, Arora et al. 1995)
Chandigarh	550	Low socioeconomic	6	(Bansal, Sehgal et al. 2004)
Chandigarh	600	General population	5.5	(Khurana, Aggarwal et al. 2005)
Chandigarh	970	Low socioeconomic	69.5	(Ramesh, Malla et al. 1991)
Chandigarh	82667	Patients - OPD	4-9	(Sethi, Sehgal et al. 2000)
Chandigarh	120	Infants with intractable diarrhea	6	(Thapa 1994)
Delhi	175	Children with persistent diarrhea	20	(Khurana, Taneja et al. 2008)
Delhi	127	Children with diarrhea	11	(Kaur, Rawat et al. 2002)
Delhi	100	Adult and children, malabsorption	24	(Behera, Mirdha et al. 2008)
Delhi	939	Urban slum dwellers	8.4	(Mirdha and Samantray 2002)
Lucknow	1071	Urban and rural population	22	(Nitin, Venkatesh et al. 2007)
Lucknow	1061	Preschool slum children	32.9	(Awasthi and Pandey 1997)
Pune	76	children <5 yr with diarrhea	7.89	(Mahendraker, Dutta et al. 1991)
Punjab	-	Preschool children	35.1	(Walia, Ganguly et al. 1986)
Srinagar	514	School children	7.2	(Wani, Ahmad et al. 2007)
South India				
Bangalore	361	Children with diarrhea	8-10 (<6 m - 2.1)	(Shetty, Narasimha et al. 1990)
Chennai	324	Rural and urban population	16- rural 22.6 - urban	(Fernandez, Verghese et al. 2002)
Karnataka	10,000	Adults	37.1	(Shenoy, Urs et al. 1998)
Karnataka	1020	Adult and children, healthy	2.45	(Subbannayya, Babu et al. 1989)
Vellore	78	Asymptomatic rural population	53.8	(Kang, Mathew et al. 1998)
Vellore	452	Children in urban slum	22.9	(Ajjampur, Sankaran et al. 2009)
East India				
Varanasi	2095	Patients with acute diarrhea	1.7	(Nath, Choudhury et al. 1999)
Bihar	326	Rural and semi-urban	28.2	(Saha, Behal et al. 1996)
Kolkata	383	Under five children with diarrhea	0.4	(Sircar, Deb et al. 1984)
Kolkata	1103	Children and adults with diarrhea	13.3	(Mukherjee, Chowdhury et al. 2009)

Kolkata	2519	Hospitalized patients with diarrhea	11.2	(Nair, Ramamurthy et al. 2010)
Sikkim	2559	General population	5.9	(Mitra 1970)

Transmission

The infective cysts are able to survive in moist and cool environment and in water for several months and are also able to resist chlorination (Wolfe 1992). Transmission occurs frequently through contaminated water and in developing countries food-borne transmission is also common. Person-to-person transmission occurs in groups with poor faeco-oral hygiene as in day-care centers for children. The infectious dose for giardiasis was found to be as low as 10-25 cysts in a human prisoner volunteer study (Rendtorff 1954). In a later study, using the gerbil model an ID50 of 100 cysts was determined (Visvesvara, Dickerson et al. 1988).

The potential for zoonotic transmission of *Giardia* has been recognized since 1979 (Thompson 2004; Caccio and Ryan 2008), though it has not been conclusively proved. Possible routes of such zoonotic transmission are from dairy cattle, from companion animals like cats and dogs or from wild life. Traub et al in a study in a remote tea-growing community in India showed a significantly high association between giardiasis in cases and the presence of a *Giardia*-positive dog in the same household (Traub, Monis et al. 2004). In another study, Equadorian children who live in households with domestic animals were 2-5 times more likely to be infected by *Giardia* compared to other children (Sackey, Weigel et al. 2003). Giardiasis is also widespread among livestock and infected calves can shed as much as 10^5-10^6 cysts per gram of feces (Thompson 2004). More recent molecular epidemiological studies in Europe and Africa

have also found evidence for cross species transmission (Gelanew, Lalle et al. 2007; Foronda, Bargues et al. 2008; Sprong, Caccio et al. 2009)

Pathogenesis

The pathogenesis of giardiasis is not completely understood. Due to the variation in the host susceptibility pattern of *Giardia* both at the species and genotype level, a relevant animal model to study the infectious process of *Giardia* in humans has been difficult to find (Eckmann 2003) and most studies have been conducted on a murine model using *Giardia muris*. This limitation has been partly overcome by *G. duodenalis* clone GS/M-H7 which can infect adult mice (Byrd, Conrad et al. 1994). However, GS/M-H7 belongs to the *Giardia duodenalis* genotype assemblage B, and due to differences between different genotypes infecting humans, information obtained from GS/M-H7 infection in mice has to be extrapolated to infections due to other genotypes as well. In contrast, the gerbil model is more relevant as different *G. duodenalis* strains can infect and cause disease symptoms in adult gerbils (Astiazaran-Garcia, Espinosa-Cantellano et al. 2000).

A wide range of pathogenic mechanisms have been attributed to giardial diarrhea including reduction in intestinal disaccharidase activity, reduction in intestinal protease activities, disruption of microvillous brush border, villus shortening or atrophy, crypt hyperplasia increased epithelial permeability, mucosal inflammation and bacterial overgrowth (Muller and von Allmen 2005) and more recently, hypermotility. Biopsy specimens have revealed that trophozoites line the villi and intervillous spaces and this orientation of the trophozoites probably

causes mechanical obstruction to absorptive process in the small intestine (Wolfe 1992). Damage to the enterocytes may lead to the deficiency of disaccharidase and subsequent osmotic diarrhea. Apoptosis of the intestinal epithelia and disruption of tight junctions may result in increased intestinal permeability (Buret, Mitchell et al. 2002; Ali and Hill 2003). In a recent study on children chronic mucosal inflammation with eosinophilic infiltrate has been found (Koot, ten Kate et al. 2009). Though no known toxin is elaborated, thiol proteinase and mannose-binding lectins may be involved in epithelial injury (Farthing 1997).

The persistence of infection and chronic disease in some patients may be explained by immunoevasion due to antigenic variation in the immunodominant cysteine-rich surface antigens, variable surface protein (VSP) (Adam 2001; Singer, Elmendorf et al. 2001; Carranza and Lujan 2010). These VSP cover the entire exterior of the trophozoites and are the main antigenic targets of the host response. Only 1 of ~ 205 VSP genes encoded by the genome is expressed at a time (Smith, Aley et al. 1998). Recent research suggests that antigen switching is regulated at the post-transcriptional level by a mechanism similar to RNA interference (Prucca, Slavin et al. 2008). The switching may also be a result of selection by the immune response or of the trophozoites to varying environmental conditions as it moves through the intestinal lumen.

Immune response

The immune response of the infected host and its role in modulating giardial infection and pathogenesis is not completely understood yet. The presence of a large number of asymptomatic infections points to an effective host immune response against the pathogen.

Innate immunity may have a major role in preventing infection by *Giardia*. The mucus layer in the intestine provides a natural barrier and prevents access to the underlying epithelia (Roxstrom-Lindquist, Palm et al. 2006). Antimicrobial peptides including α-defensin and lactoferrin have been shown to have inhibitory effect to *Giardia* (Eckmann 2003; Roxstrom-Lindquist, Palm et al. 2006). Another antimicrobial product which may provide mucosal defense against *Giardia* is inducible nitric oxide (iNO) which was found to inhibit proliferation of trophozoites in vitro and also inhibited encystation and excystation (Eckmann, Laurent et al. 2000). However, *Giardia* can evade this inhibition by competitive utilization of arginine as well as by the presence of A-type flavoprotein which has NO-reductase activity (Eckmann, Laurent et al. 2000). In view of *Giardia* being a microaerophilic parasite with limited capacity to neutralize reactive oxygen species (ROS), ROS produced by the intestinal epithelial cells may be detrimental for the trophozoites (Roxstrom-Lindquist, Palm et al. 2006). IL-6 secreted by mast cells has also been shown to be necessary for control of early acute infection (Zhou, Li et al. 2003). Complement mediated killing by trophozoites via activation of the lectin pathway has also been seen (Evans-Osses, Ansa-Addo et al. 2010). With reference to TLR signaling, dendritic cells (DC) incubated with giardial extract and TLR ligands resulted in suppression of CD80, CD86 and MHC-II molecules and IL-12, while IL-10 production was enhanced. This is shown to be mediated by the inhibition of phosphoinositide 3-kinase (PI3K) pathway. The authors have concluded that *Giardia* interferes with the innate response such that the host response is sufficient to control infection but is not inflammatory in nature (Kamda and Singer 2009).

In the adaptive immune response, both T-cells and B-cells are involved in mounting an effective immune response against *Giardia*. T-cell deficient mice infected with *G.muris* and *G.*

duodenalis failed to clear the infection and developed chronic giardiasis (Singer and Nash 2000). It was also shown that αβ-TCR-bearing T-cells are needed for the control of infection while γδ-TCR-bearing T-cells are not (Singer and Nash 2000). In a study to elucidate T-cell stimulating antigens of *Giardia* using *Giardia* specific T cell hybridomas and assaying the IL-2 response after activation, Astiazaran-Garcia et al found that among the soluble proteins extracted, 40-64 kDa, 65-77 kDa and 90-110 kDa proteins had a stimulating effect on hybridomas 9B10, 4D5 and 10G5 respectively (Astiazaran-Garcia, Quintero et al. 2009). These antigens had comparable molecular masses to those found by other workers as being recognized by anti giardial IgG and IgA (Palm, Weiland et al. 2003; Tellez, Palm et al. 2005; Velazquez, Beltran et al. 2005; Davids, Palm et al. 2006). The stimulated hybridomas had phenotypic markers characteristic of T helper cells, and thus these antigens may potentially be recognized by T-helper cells in-vivo leading to activation of B cell clones.

B-cell response in the form of secretory IgA and IgM play an important role in eradication of *Giardia*. Many antigens have been characterized which are recognized by anti-giardial antibody, of which the variant-specific surface protein (VSP) is the most well characterized (Eckmann 2003). Production of specific anti-*Giardia* antibody in the mucosa of IgA isotype has been shown to correlate with clearance of infection in the *Giardia muris* mouse model (Snider and Underdown 1986). In human studies, secretory IgA antibodies against membrane antigens are found in saliva of infected individuals but not in healthy individuals (Rosales-Borjas, Diaz-Rivadeneyra et al. 1998). Secretory IgA antibodies in breast milk of lactating mothers have also been proved to be protective against *Giardia* infection in infants (Morrow, Reves et al. 1992; Mahmud, Chappell et al. 2001; Tellez, Palm et al. 2005). These

antibodies recognized up to 16 different giardial proteins lying between 20-165kDa molecular weight, of which the immunodominant protein was that of the VSP. Mice with deficient IgA production cannot eradicate infections but over an extended period were able to reduce the initial infectious load. Thus while IgA plays a role in eradication of the infection, other mechanisms are also necessary for host defence (Langford, Housley et al. 2002). In a gerbil model, both systemic as well as intestinal humoral response correlated with the control of infection (Amorim, Silva et al. 2010). Recently, studies have found that immune dependent (probably secretory IgA) hypermotility, although potentially a mechanistic cause of diarrhea, could also be associated with clearance of infection and therefore constitute a part of the host defense (Andersen, Gillin et al. 2006).

Clinical manifestations

The majority of infected individuals with *Giardia* in stools remain asymptomatic (35-70%) (Mandell, Bennett et al. 2005). Some develop acute diarrheal illness with fever, epigastric pain, nausea and vomiting, and rarely giardiasis can result in explosive diarrhea with bloody stool. Chronic giardiasis manifests as malaise, abdominal bloating and discomfort, intermittent mild diarrhea, and malabsorption. In a prospective study, *Giardia* infection was identified in 6.5% of patients with irritable bowel syndrome and dyspepsia (Grazioli, Matera et al. 2006). In another study, 44% of patients who attended an endoscopy unit due to dyspeptic symptoms had *Giardia* infection (Yakoob, Jafri et al. 2005). Allergic skin manifestations including urticaria and angioedema are also known to be associated with giardiasis. The wide spectrum of symptomatology of giardial infection has been attributed to various factors such as inoculum

size, specific host response and parasitic factors of which genotypic difference may be an important determinant (Wolfe 1992).

The consequence of giardial infection whether symptomatic or asymptomatic, is more significant in infants and children. In a study by Fraser et al in Israeli Bedouin infants, where the infants were followed from birth to ~ 2 years of age, z scores for weight-for-age and weight-for-height were significantly different between those infants who were never positive for *Giardia* and those that were *Giardia* carriers, and this growth faltering was subsequent to *Giardia* infection even though infection did not always cause diarrhea (Fraser, Bilenko et al. 2000). Stunting due to giardiasis has also been observed in school going children (Al-Mekhlafi, Azlin et al. 2005; Nematian, Gholamrezanezhad et al. 2008) and treatment of giardiasis resulted in increased weight and height gain, allowing catch-up growth in pre-school children in rural Guatemala (Gupta and Urrutia 1982). Psychomotor development is also adversely affected by giardial infection (Simsek, Zeyrek et al. 2004). In children followed up from birth to 2 years of age, when administered the Wechsler intelligence scale for children-revised (WISC-R) at 9 years of age, it was found that the scores for children with >1 episode of giardial diarrhea were on an average 4.1 points lower than those who had ≤1 episode per year (Berkman, Lescano et al. 2002).

The cause of poor growth and nutritional status due to giardiasis has also been studied. Children with giardiasis have a lower mean hemoglobin levels than uninfected children and treatment results in improvement of the hemoglobin levels (Jimenez, Rodriguez et al. 1999; Sackey, Weigel et al. 2003; Monajemzadeh and Monajemzadeh 2008). In a study in Mexico,

giardiasis was also significantly associated with vitamin A deficiency in children (Quihui-Cota, Astiazaran-Garcia et al. 2008). There are however, some studies that have failed to show any association between giardiasis and nutritional/growth parameters in children (Lunn, Erinoso et al. 1999; Hollm-Delgado, Gilman et al. 2008).

Diagnosis

Traditional diagnosis of giardiasis is by ova and cyst examination of stool and it is recommended to examine three stool samples for the diagnosis. Though the cyst is the morphological form in which the organism is passed, freshly passed stool in patients with explosive diarrhea can have trophozoites which can be demonstrated in the saline preparation. In a study by Hiatt et al, 88% of giardiasis was diagnosed by a single stool examination from symptomatic cases in a medical care program and there was an increase in the yield by 11.3% when three samples are examined (Hiatt, Markell et al. 1995). However in a study on a rural asymptomatic population in Vellore, sensitivity of a single stool sample examination was less than 40% and that of three samples was nearly 80%. The optimum number of samples for detection of intestinal parasites in this asymptomatic population was eight samples (Kang, Mathew et al. 1998).

Antigen detection assays using either an enzyme immune assay or direct immunofluorescence are available for diagnosis of giardiasis. Most immunological methods utilize the *Giardia* specific antigen 65 (GSA65) which is a glycoprotein present in the cyst wall (Aziz, Beck et al. 2001). Sensitivity of EIA ranges from 94-99% with 100% specificity while immunofluorescence assays have 100% sensitivity and specificity (Garcia and Shimizu 1997). In

cases of difficult diagnosis the string test or the entero-test can be done to sample duodenal contents for microscopy. A gelatin capsule attached to a string (90 cm for children and 140 cm for adults) composed of silicon rubber covered thread and soft nylon yarn is swallowed by the patient. A small weight attached to the nylon yarn helps to carry the string into the duodenum while the free end of the string is taped on the patient's cheek. After 4 hours the string is pulled out and the mucus sticking on the thread is examined for trophozoite forms (Thomas, Goldsmid et al. 1974). Duodenal aspiration and biopsy can also be done for the demonstration of the trophozoites, but is invasive. Demonstration of anti-*Giardia* antibodies is not useful for diagnosis of giardiasis as IgG titer remain high for prolonged intervals and the utility of IgM in differentiating current from past infection is not clear (Granot, Spira et al. 1998).

Treatment and Prevention

Metronidazole has long been the drug of choice for the treatment of giardiasis. This nitroimidazole gets activated by parasitic ferridoxin and binds to the parasitic DNA, causing damage and subsequent death of the trophozoites (Samuelson 1999). Nitazoxanide, a pyruvate ferridoxin, is a newer drug approved for the treatment of pediatric giardiasis and interferes with energy production of the parasite (Halsey 2009). Its efficacy with a 3 day dose is comparable to metronidazole but with fewer side-effects (Ortiz, Ayoub et al. 2001; White 2004). More recently, a meta- analysis of 8 randomized control trials (RCTs) revealed that albendazole was as effective as metronidazole for the treatment of giardiasis in humans and had fewer side effects (Solaymani-Mohammadi, Genkinger et al. 2010). In the light of poor compliance and reports of drug resistance to metronidazole (Upcroft and Upcroft 1993), albendazole may be considered a

safe, effective alternative. Other drugs reported to be effective in treating giardiasis include - quinacrine, furazolidone, paromomycin and bacitracin (Gardner and Hill 2001).

Good personal hygiene and proper treatment of water supply are essential for the prevention of giardiasis. Since the cysts are resistant to chlorination, water supply needs to go through a process of sedimentation, flocculation and filtration. In the west, outbreaks in nurseries and day cares can be prevented by good hand hygiene and treatment of symptomatic children (Bartlett, Englender et al. 1991). In the developing world, provision of safe water, health education and segregation of feces with sanitary toilets will go a long way in preventing transmission.

A vaccine is available for the prevention of giardiasis and to decrease cyst shedding in dogs (GiardiaVax™) (Olson, Ceri et al. 2000). The vaccine consists of inactivated trophozoites (Anderson, Brooks et al. 2004) and is given in two doses subcutaneously 2 to 4 weeks apart. Puppies and kittens challenged orally with *Giardia* cysts 6 months and 1 year after vaccination showed fewer symptoms, did not lose weight, had lower cyst burdens and if symptomatic, were ill for a shorter duration than the control animals post – challenge (Olson, Ceri et al. 2000). Another suggested use of this vaccine is as an immunotherapeutic agent for animals with chronic giardiasis and treatment failure (Olson, Hannigan et al. 2001). However another study found no difference in cyst shedding between the use of antimicrobial agents along with vaccination and use of antimicrobial agents alone for treatment of naturally acquired giardiasis in dogs (Payne, Ridley et al. 2002). Anderson et al, similarly found no decrease in cyst shedding after vaccination in asymptomatic infections in dogs (Anderson, Brooks et al. 2004). In spite of these

negative findings, the potential for zoonotic transmission of giardiasis makes the case for vaccination of animals, especially companion animals to prevent spread to humans. More recent studies have found that primary infection with altered trophozoites in which the antigenic (VSP) variation mechanism had been disrupted could protect from subsequent infections in the gerbil model (Rivero, Saura et al. 2010).

Zymodemes and Genotypes

Heterogeneity among *G. duodenalis* was first shown by zymodeme analysis or multilocus enzyme electrophoresis of axenised trophozoites. Isolates having the same enzyme profile for a set of enzymes were referred to as comprising a *zymodeme*. The enzymes used for the analysis were esterase, glucose-6-phosphate dehydrogenase, hexokinase, glutamate-oxaloacetate transaminase, glucose phosphate isomerase, malate dehydrogenase, malic enzyme, phosphoglucomutase, nucleoside phosphorylase, 6-phosphogluconate dehydrogenase (Meloni, Lymbery et al. 1988). Nash, Homan and Mayrhofer in independent studies, described two major groups infecting humans by restriction-endonuclease analysis (Nash, McCutchan et al. 1985; Homan, van Enckevort et al. 1992; Mayrhofer, Andrews et al. 1995). Subsequent studies comparing the small-subunit rRNA (SSU rRNA), triosephosphate isomerase (tpi) and glutamate dehydrogenase (gdh) genes confirmed the existence of two major genotypes among the human isolates – named assemblage A and B (Homan, Gilsing et al. 1998; Lu, Wen et al. 2002).

The whole genome of both these assemblages have been mapped using laboratory adapted axenised strains (Smith, Gillin et al. 1982; Adam 2001) (WB- assemblage A and GS - assemblage B) and have a 77% ± 5% nucleotide homology and 78% ± 14% amino acid

homology (Franzen, Jerlstrom-Hultqvist et al. 2009) (http://giardiadb.org/giardiadb/). A higher level of allelic sequence diversity (average 0.5%) was observed in the GS isolate than in the WB isolate (<0.01% divergence). The VSP repertoires of GS were also found to be very different from that of WB isolate (Franzen, Jerlstrom-Hultqvist et al. 2009). Whether these differences between assemblage A (WB) and assemblage B (GS) genomes extend to clinical or field isolates remains to be studied. Further work using molecular tools then revealed additional host specific assemblages (C to G) with assemblage C and D described in dogs, assemblage E in cattle and pigs, assemblage F in cats and assemblage G in rodents (Adam 2001).

Assemblage A and B isolates infecting humans have been further divided into sub-genotypes AI and AII, and BIII and BIV according to restriction digestion of PCR products at the glutamate dehydrogenase (gdh) (Monis, Mayrhofer et al. 1996; Read, Monis et al. 2004) and the trios-phosphate (tpi) (Amar, Dear et al. 2002) locus. Sequence analysis at the β-giardin gene has also been used as a tool to sub-genotype isolates into AI, AII and AIII and assemblage BI, BII, BIII and BIV (Caccio, De Giacomo et al. 2002). AIII was found to affect only wild animals and never seen in humans. Intra assemblage variation at the β-giardin locus resulted in classification into 8 sub-genotypes (A1-A8) within assemblage A and 6 sub-genotypes (B1-B6) within assemblage B (Lalle, Pozio et al. 2005). These methods have been extensively applied in epidemiological studies on giardiasis and are summarized in Table 2. Comparison of gene target for genotyping has shown a higher sensitivity for tpi than gdh, and while gdh has a better resolution and is able to sub-type assemblage B (Bertrand, Albertini et al. 2005). Sequence heterogeneity at the tpi, β-giardin and gdh loci for assemblage B has also been reported (Caccio, Beck et al. 2008).

Some workers have stated that variation in genotypes, both phenotypic and host specificity, warrants the classification of these genotypes to separate species (Monis, Caccio et al. 2008). Thus, assemblage C/D infecting dogs and other canids have been proposed to be placed under the new species name *G. canis*; assemblage F infecting cats under the new species *G. cati*; assemblage E infecting cattle and other hoofed livestock under new species *G. bovis*; and assemblage G infecting rodents in the new species *G. simondi* (Monis, Caccio et al. 2008). Further, considerable differences between the genotypes assemblage A and assemblage B infecting human has also raised questions about their separation into two different species, *G. duodenalis* for assemblage A and *G. enterica* for assemblage B (Monis, Caccio et al. 2008). Currently, *G. lamblia* is now considered to encompass a species complex rather than a single species (Monis, Andrews et al. 1999; Monis, Andrews et al. 2003).

Table 2: Genetic Loci and Molecular Tools for Genotyping of *Giardia* spp.

Locus	Method	Genotypes	Reference
Tpi	PCR/PCR-RFLP	Assemblage A and B; *Rsa I* RE of assemblage A: AI, AII	(Amar, Dear et al. 2002)
Gdh	PCR-RFLP	Assemblage A I and A II Assemblage B III and B IV	(Monis, Mayrhofer et al. 1996)
Gdh	PCR-RFLP	Nla IV RE: Assemblage AI, AII, B, C, D,E *Rsa I* RE of assemblage B: BIII, BIV	(Read, Monis et al. 2004)
β-giardin	PCR-sequencing	Assemblage A1, A2, A3, B1, B2, B3, B4	(Caccio, De Giacomo et al. 2002)
	PCR-RFLP	*Hae III* RE: assemblage A, B , E *Hha I* RE assemblage A: A1, A2/3	
β-giardin	PCR-sequencing	Assemblage A 8 subgenotypes (A1-8) Assemblage B 6 subgenotypes (B1-6) Assemblage D 2 subgenotypes (D1-2) Assemblage E 3 subgenotypes (E1-3)	(Lalle, Pozio et al. 2005)
IGS rDNA	PCR	Assemblage A1, A2 and B	(Lee, Lee et al. 2006)
18S rRNA	PCR-sequencing	Group 1, 2	(Hopkins, Meloni et al. 1997)
18S rRNA	Multiplex qPCR (Scorpion probes)	Assemblage A and B	(Ng, Gilchrist et al. 2005)
5.8S rDNA and ITS1&2	PCR-sequencing	All seven *G. lamblia* assemblages, *G. ardeae, G. muris* and *G. microti*	(Caccio, Beck et al. 2010)

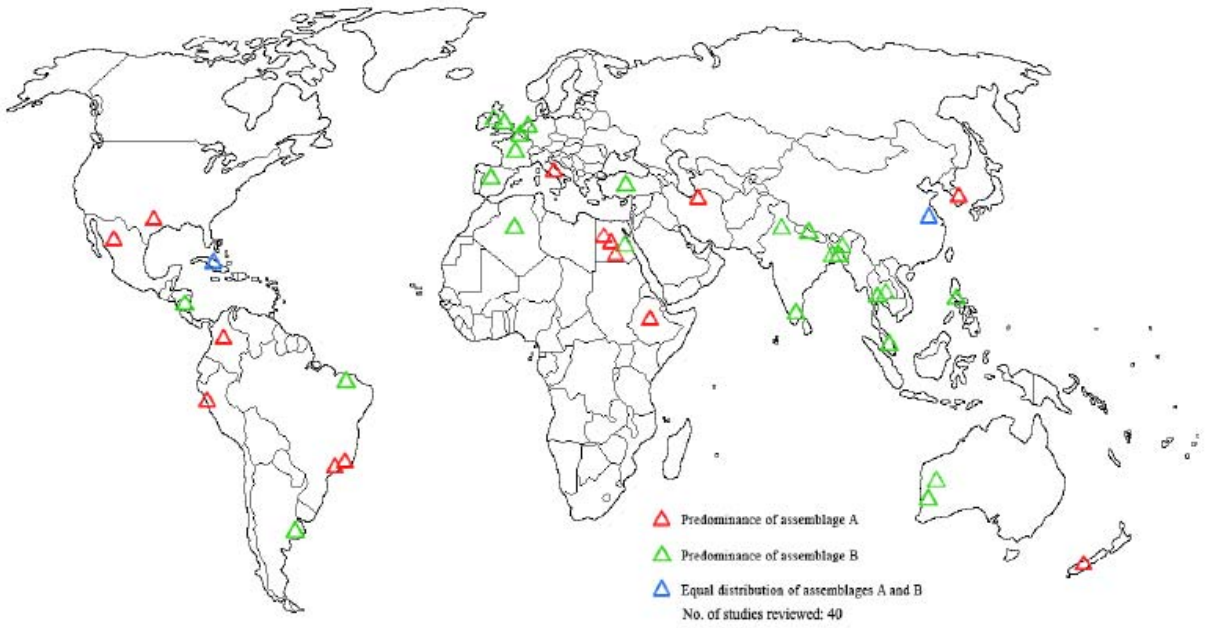
Molecular Epidemiology

Molecular epidemiological studies with genotyping of isolates from different parts of the world have shown varying distribution of the human associated genotypes, and generally a particular genotype is found to predominate in a particular area (Figure 2). Mixed infection with different assemblages or with different subtypes of same assemblage are also known (Kohli, Bushen et al. 2008; Helmy, Abdel-Fattah et al. 2009; Lalle, Bruschi et al. 2009; Breathnach, McHugh et al. 2010). In most of the epidemiological studies done in Asia including from Bangladesh, Philippines, Thailand, Nepal, Malaysia and India, a predominance of the assemblage B has been documented (Haque, Roy et al. 2005; Ng, Gilchrist et al. 2005; Yason and Rivera 2007; Ajjampur, Sankaran et al. 2009; Mohammed Mahdy, Surin et al. 2009; Singh, Janaki et al. 2009; Kosuwin, Putaporntip et al. 2010; Tungtrongchitr, Sookrung et al. 2010). A study from Iran and South Korea showed predominantly assemblage A while a single study from China showed equal distribution of both assemblage A and B (Park, Yong et al. 1999; Yong, Park et al. 2000; Lu, Wen et al. 2002; Babaei, Oormazdi et al. 2008). However, the number of isolates characterized in the studies from Korea and China were very small (5-10 in number).

Previous reports from Africa including 3 studies from Egypt and one from Ethiopia showed a predominance of assemblage A (El-Shazly, Mowafy et al. 2004; Gelanew, Lalle et al. 2007; Abdel-Moneim and Sultan 2008; Helmy, Abdel-Fattah et al. 2009). However in more recent studies from Egypt and Algeria, a higher prevalence of assemblage B was found (Foronda,

Bargues et al. 2008; Lalle, Bruschi et al. 2009). In Europe, studies from Spain, United Kingdom, Belgium, Turkey, France and Netherlands showed a predominance of assemblage B (Amar, Dear

Fig. 2 - World-wide distribution of giardial assemblages



et al. 2002; Aydin, Besirbellioglu et al. 2004; Bertrand, Albertini et al. 2005; van der Giessen, de Vries et al. 2006; Sahagun, Clavel et al. 2008; Geurden, Levecke et al. 2009; Breathnach, McHugh et al. 2010) while two studies done in Italy show a predominance of assemblage A (Caccio, De Giacomo et al. 2002; Lalle, Jimenez-Cardosa et al. 2005). In the Americas, there seem to be pockets of areas with differing predominant genotypes. Studies done in Mexico, Columbia and Peru showed a predominance of assemblage A(Ravid, Duque et al. 2007; Eligio-Garcia, Cortes-Campos et al. 2008; Perez Cordon, Cordova Paz Soldan et al. 2008). Nicaragua and Argentina, on the other hand had assemblage B as the predominant organism (Lebbad, Ankarklev et al. 2008; Minvielle, Molina et al. 2008). In Brazil, samples from the southern states of Sao Paulo and Rio de Janeiro showed a higher prevalence of assemblage A and those from the northeastern state of Ceará show more of assemblage B (Souza, Gennari et al. 2007; Volotao, Costa-Macedo et al. 2007; Kohli, Bushen et al. 2008). Cuba, on the other hand, had an equal prevalence of both genotypes (Pelayo, Nunez et al. 2008). A study done in Texas showed predominance of assemblage A, similar to that of neighboring Mexico (Hussein, Yamaguchi et al. 2009). Australia showed a predominance of assemblage B while neighboring New Zealand showed predominance of assemblage A (Winkworth, Learmonth et al. 2008; Yang, Lee et al. 2010).

Genotyping has also been carried out on animal samples to identify potential zoonotic sources of infection. Though infection in cattle is mainly by livestock genotype assemblage E, a small proportion (<20%) can harbor assemblage A which may be transmissible to humans

(Thompson 2004). The role of companion animals in zoonotic transmission may be also be significant as genotypes affecting humans have been isolated from pet cats and dogs frequently (Traub, Monis et al. 2004; Lalle, Jimenez-Cardosa et al. 2005; Souza, Gennari et al. 2007). In turn, some studies have identified zoonotic genotypes in humans. The cat associated assemblage F has been detected in mixed infections along with assemblage A from humans in Ethiopia (Gelanew, Lalle et al. 2007), cattle associated assemblage E in 15% of giardial samples in Egypt (Foronda, Bargues et al. 2008). Occasional assemblage C, D, E and F infections in humans was also reported in a study from Europe (Sprong, Caccio et al. 2009). In this study, Sprong et al found that assemblage B was more human restricted while assemblage A was less restricted and was present at comparable rates in humans, companion animals, livestock and wildlife.

This heterogeneity in the distribution of the genotypes around the world is important to document as genotype may influence clinical manifestations and the transmission ecology of the parasite. Genotype linked differences in symptomatology of giardiasis was first described in Dutch patients where mild intermittent disease was associated with assemblage A and more severe persistent disease with assemblage B (Homan and Mank 2001). Since then, some workers have found an association of diarrhea with assemblage B (Gelanew, Lalle et al. 2007; Mohammed Mahdy, Surin et al. 2009; Tungtrongchitr, Sookrung et al. 2010) while other workers have found an association with assemblage A (Read, Walters et al. 2002; Aydin, Besirbellioglu et al. 2004; Perez Cordon, Cordova Paz Soldan et al. 2008). Some studies however, found no correlation between symptomatology and the infecting genotype (Lalle, Pozio et al. 2005; Abdel-Moneim and Sultan 2008; Kohli, Bushen et al. 2008; Lebbad, Ankarklev et al. 2008) (Table 3).

Three genotyping studies that have been carried out in India till date in Delhi, Assam and Vellore (Paintlia, Descoteaux et al. 1998; Traub, Monis et al. 2004; Ajjampur, Sankaran et al. 2009). The first study by Paintala et al (1998) was carried out on young adults from Delhi with and without diarrhea and an association of symptoms with assemblage A was seen. Traub et al carried out an epidemiological study on a remote tea tribe in Assam and found some evidence of zoonotic transmission from dogs. The Vellore study on children with and without diarrhea found that a majority of the isolates were of assemblage B but infection with assemblage A was more strongly associated with development of diarrhea (Ajjampur, Sankaran et al. 2009).

The present study aimed to use *three different genotyping methods* to identify the assemblages associated with diarrhea in *both children and adults in this region*. The sensitivity of the 3 genotyping methods and any genetic heterogeneity between the loci was evaluated.

Table 3: Association of Giardial Assemblage with Diarrheal Symptoms

Country	Age group	Locus	Inference	Reference
Australia	< 5 years	SSU rRNA	Assemblage **A** - 26 times more likely in diarrhea	(Read, Walters et al. 2002)
Bangladesh	All	tpi	Assemblage **A** -higher odds ratio of diarrhea Assemblage B more prevalent, heavier infections	(Haque, Roy et al. 2005)
Cuba	School children	β-giardin, gdh	Assemblage **B** - significantly associated with symptomatic disease	(Pelayo, Nunez et al. 2008)
Egypt	All	tpi	Assemblage **A** – intermittent, severe symptoms Assemblage B - mild persistent disease	(Helmy, Abdel-Fattah et al. 2009)
England	All	tpi &SSU rRNA	Assemblage A infection - more frequent fever	(Breathnach, McHugh et al. 2010)
Ethiopia	All	β-giardin, tpi, gdh & SSU rRNA	Assemblage **B** stronger correlation with diarrhea	(Gelanew, Lalle et al. 2007)
India	< 3 years	tpi	Assemblage A - single or dual infection - diarrhea more frequent	(Ajjampur, Sankaran et al. 2009)
Malaysia	All	SSU rRNA	Assemblage **B**- higher risk of signs and symptoms	(Mohammed Mahdy, Surin et al. 2009)
Netherlands	All	gdh	Assemblage A - mild disease; Assemblage **B** - severe disease	(Homan and Mank 2001)
Peru	< 9 years	gdh	Assemblage **A** - diarrhea ; Assemblage B - asymptomatic	(Perez Cordon, Cordova Paz Soldan et al. 2008)
Spain	< 5 years	tpi	Assemblage **A II** associated with symptomatic in children < 5 yr	(Sahagun, Clavel et al. 2008)
Thailand	All	SSU rRNA, β-giardin, gdh & tpi	Assemblage **B III** associated 70% with symptomatic disease either as single infection or with AI or AII	(Tungtrongchitr, Sookrung et al. 2010)
Turkey	All	tpi	85% of symptomatic- assemblage **A** 92% of asymptomatic - assemblage B	(Aydin, Besirbellioglu et al. 2004)

MATERIALS AND METHODS

Study Subjects

The study subjects enrolled were children with diarrhea, children with no diarrhea and adults with gastrointestinal symptoms. The study was approved by the Institutional Review Board of Christian Medical College, Vellore. Informed consent was obtained from parents of children enrolled in the study. A waiver of consent was granted by the Institutional Review Board for the adult samples as they were taken from routine diagnostic work without patient identifiers. The inclusion criteria were -

1. Children under the age of 6 years presenting with diarrhea in the community
2. Children without diarrhea under the age of 6 years in the community
3. Adults (>18 years of age) with gastrointestinal symptoms attending the hospital for whom a stool routine examination for ova and cysts was requested
4. *Giardia* cyst and/or trophozoites seen in stool microscopy

Since this was a pilot study aimed at determining the common assemblages circulating in the community, an arbitrary sample size of 25 *Giardia* positive cases was included in each group.

Children with diarrhea in the community: Children with giardial diarrhea were recruited from among a birth cohort of children established to study diarrheal diseases (Gladstone, Muliyil et al. 2008) and had been followed up twice-weekly up to the age of 3 years between January 2002

and April 2006. The study setting comprised of three adjacent urban slums in Vellore with an area of around 2.2 km^2 and population of approximately 33,390. Children with diarrhea were assessed clinically, and details of the number of stools passed per day, any associated fever or vomiting, and treatment given was recorded daily until the cessation of diarrhea. An episode of diarrhea was defined as at least 1 day of diarrhea (three loose stools in a 24 hour period) followed by at least 2 days without diarrhea (Newman, Moore et al. 2001). Acute diarrhea was defined as fewer than 4 days and persistent diarrhea as more than 14 days. Children with diarrhea more than 3 days but fewer than 14 were described as indeterminate. A relapse of giardial diarrhea was defined as a second episode commencing between 2 and 7 days after the conclusion of the original *Giardia* diarrhea and a recurrence was defined as infection occurring more than 7 days after the initial *Giardia* diarrhea (Newman, Moore et al. 2001).

Children without diarrhea in the community: Children with asymptomatic giardial infections diagnosed by stool microscopy, who lived in the same urban slum area and attended the urban health center in the area for illnesses not related to the gastrointestinal tract between March and July 2008 were included as controls.

***Adults with gastrointestinal symptoms*:** Adults (>18 years of age) with gastrointestinal symptoms attending the hospital for whom a stool routine examination for ova and cysts was requested were included between September and October 2008. The hospital charts of the adult patients were reviewed retrospectively after the completion of genotyping, for the socio-demographic profiles, signs and symptoms, and clinical diagnosis. Gastrointestinal symptoms for

the adult subjects were defined as having any one or combination of the following symptoms or clinical diagnosis of dyspepsia and/or functional bowel disorder: diarrhea, malaise, flatulence, foul smelling, greasy stool, abdominal cramps, bloating, anorexia, nausea, weight loss, vomiting, fever and constipation.

Sample collection

Stool samples were collected in sterile wide-mouthed containers and transported to the laboratory immediately. Each stool sample was given a unique identification number and stored in aliquots at -70° C and 4°C.

Laboratory methods

***Stool microscopy*:** All samples on receipt in the laboratory were screened for ova and cysts by microscopic examination of saline and iodine preparations. Samples positive for *Giardia* cysts and/or trophozoites were included in this study.

***DNA extraction*:** DNA was extracted from the stool samples using the QIAamp DNA Stool Mini Kit (Qiagen Cat No. 51504) as per the manufacturers' protocol. The following steps were included:

1. A peanut sized amount/200uL of stool sample was transferred to labeled microcentrifuge tubes and 900µl of ASL buffer was added. The mixture was homogenized by vortexing for 1 min and then incubated in the water bath at 95°C for 5 min. After the incubation, the tube was vortexed for 15 seconds and centrifuged at full speed (3200rpm) for 2 min. The supernatant was transferred into new microcentrifuge tube and the pellet discarded.

2. One inhibitEX tablet was added to the supernatant and vortexed immediately for 1 min. and then incubated further at room temperature (24-25°C) for 1 min. The tube was centrifuged for 4 min., the supernatant transferred to new microcentrifuge tube and again centrifuged for 4 min.
3. Fifteen µl of proteinase K was pipetted into a microcentrifuge tube and to this 200µl of the supernatant from the above step is added. Buffer AL (200 µl) was added into the same tube and vortexed for 15 seconds. The mixture was incubated at 70°C for 10 min. Following incubation, the tube was centrifuged briefly to remove drops from the inside of the tube lid. Ethanol (100%, 200 µl) was added to the lysate, mixed by vortexing and centrifuged briefly.
4. The lysate was added into labeled spin column placed in a collection tube and centrifuged for 1 min. The filtrate was discarded, 500µl of AW1 buffer added into the column and centrifuged for 1 min. The filtrate was again discarded, 500µl of AW2 buffer added into the column and centrifuged for 3 min. The column was placed in a new collection tube and centrifuged again to remove any remaining AW2 buffer.
5. The column was transferred to new microcentrifuge tube and 200µl of AE buffer added into the column. The column was kept at room temperature for 1 min and centrifuged for 1min to elute out the DNA. The spin column was discarded and DMSO is added to make up 10% of the eluate and then the extract is stored at -70°C.

Positive and Negative controls: Stool samples previously identified to have specific *Giardia* assemblages were used as positive controls for the study. Sterile water was used as negative

control for each batch of extraction, and the extract is also used as negative control for the follow up PCR steps.

PCR-RFLP for genotyping

Tpi gene PCR-RFLP: The tpi gene of the organism was amplified in a nested PCR reaction using primers and conditions as described by Amar et al (Amar, Dear et al. 2002) and had been standardized previously in the laboratory (Ajjampur, Sankaran et al. 2009) (Table 4). The first reaction was a duplex PCR in a final volume of 20 µL containing 10µl DNA extract, 1X PCR buffer, 2mM $MgCl_2$, 0.3µM of each primer, 0.25mM of each deoxynucleotide phosphate, 0.75 µL of *taq* DNA polymerase and 0.5µl DMSO. The following cycling parameters were used for amplification: 94°C for 1 min; 55 cycles of 94°C for 20 sec, 52°C for 30 sec, 72°C for 60 sec; followed by final extension at 72°C for 5 min. The second reaction was carried out separately for assemblage A and assemblage B in separate tubes. The reaction was carried out in a mixture containing 1X PCR buffer, 0.25 mM of each deoxynucleotide phosphate, 1µM of each primers, 1.5 mM $MgCl_2$, 0.75µL of *taq* DNA polymerase and 0.5µL of DMSO in final reaction volume of 20µL. Ten µL of the 1^{st} round product, diluted 10 times and 20 times respectively for assemblage A and B reaction were used as templates. The following cycling parameters were used for amplification: 94°C for 1 min; 33 cycles of 94°C for 20 sec, 58°C for 30 sec, 72°C for 1 min; followed by final extension at 72°C for 5 min. The PCR products were detected by running in 2% agarose gel stained with ethidium bromide, along with 100-bp molecular ladder at 100 Volts. Once the product was determined to be assemblage A, restriction fragment analysis was done by digesting the product with *RsaI* to determine the subtype of assemblage A. The reaction was carried out in final volume of 30µL with 8µL of PCR product and 5 units of enzyme in 1X buffer

and incubated at 37°C for 1 hour. The restriction fragments were detected by running in 2.5% agarose gel with ethidium bromide at 70 Volts.

Gdh-PCR RFLP: The gdh gene of *Giardia* was amplified in a hemi-nested reaction using the primers and protocols described by Read et al (Read, Monis et al. 2004) (Table 4), with slight modifications and was standardized using in-house *taq* DNA polymerase. The first reaction was conducted in 25 µL volume with 1µL DNA extract, 1.5 mM $MgCl_2$, 200µM of deoxynucleotide phosphate, 12.5 ρmol of each primer (GDHeF and GDFiR), 0.5 µL DMSO and 2 µL *taq* DNA polymerase. The following cycling condition was used: one cycle of 94°C for 2 min, 56°C for 1 min, 72°C for 2 min; 55 cycles of 94°C for 30 sec, 56°C for 20 sec, 72°C for 45 sec; followed by final extension at 72°C for 7 min. The second round reaction was standardized using the same reaction volume and concentrations with the primers GDHiF and GDHiR, and 1µL of the 1st round product as DNA template, using the same cycling parameters. The final PCR product was detected by running in 1% Agarose gel with ethidium bromide and 100-bp molecular marker at 100 Volts. RFLP analysis was done to determine the assemblages AI, AII, BIII & IV, C, D and E by *Nla IV* digestion. Assemblage BIII and BIV were also discriminated by digestion with *Rsa I.* The reaction was standardized in a final volume of 20 µL with 2 units of enzyme, 3 µL of buffer, 2 µL of PCR product. The mixture was incubated at 37°C for 3 hours and the restriction fragments are detected by running in 2.5% Agarose gel with ethidium bromide at 70 Volts along with 100-bp molecular marker.

β-giardin-PCR RFLP: The β-giardin gene was amplified in a nested PCR reaction using the primers and protocol described by Lalle et al (Lalle, Pozio et al. 2005) (Table 4), with slight

modifications and standardized using the in-house *taq* DNA polymerase. The first reaction was done in a final volume of 50 µL of mixture containing 1X PCR buffer, 1.5 mM $MgCl_2$, 200 µM deoxynucleotide phosphate, 10ρmol of each primer, 2µL of *taq* DNA polymerase, 0.5 µL of DMSO and 2 µL of DNA extract. The following cycling parameters were used: 94°C for 5 min; 40 cycles of 94°C for 30 sec, 65°C for 30 sec and 72°C for 60 sec; followed by final extension at 72°C for 7 min. The second reaction was done at final volume of 50 µL with 1X PCR buffer, 1.5 mM $MgCl_2$, 200 µM deoxynucleotide phosphate, 10 ρmol of each primer, 2µL of *taq* DNA polymerase, 0.5µL of DMSO and 1 µL of 20 times diluted first round product as template. The following cycling parameters were used: 95°C for 15 min; 35 cycles of 95°C for 30 sec, 55°C for 30 sec and 72°C for 60 sec; followed by final extension at 72°C for 7 min. The final PCR product was detected by running in 1% agarose gel with ethidium bromide and 100-bp molecular marker at 100 Volts. *Hae III* restriction of the PCR product was done using 10 units of enzymes and 8µL PCR product in a final volume of 20µL. The mixture was incubated at 37°C for 1 hour and the restriction fragments were detected by running in 3% Agarose gel with ethidium bromide and 50-bp molecular marker at 70 Volts.

***Sequencing and Analysis*:** In order to confirm and validate the PCR-RFLP results, the amplified product of each representative assemblage obtained by the 3 different PCRs was sequenced (MWG Biotech Pvt Ltd, Bangalore) and the sequences obtained were compared with those available in GenBank (http://www.ncbi.nlm.nih.gov/) by a BLAST search (Altschul, Gish et al. 1990).

Data entry and Statistical Analysis

The retrieved data along with the genotyping results was entered into Microsoft Excel sheet and SPSS 16.0 software for further analysis. Categorical outcomes like sex, socio-economic status etc. were tested for significance by using the chi-square test and continuous outcomes like number of episodes of diarrhea, age etc. were tested for significance using the t-test.

Table 4: PCR Loci and Primers used to Determine Assemblage Type

Target gene	PCR step	Primers	PCR product(bp)	Restriction enzyme & products(bp)	Reference
tpi	AB duplex	TPIAF: CGA GAC AAG TGT TGA GAT GC TPIAR: GGT CAA GAG CTT ACA ACA CG TPIBF: GTT GCT CCC TCC TTT GTG C TPIBR: GGC CTT GCG TTC ATC CAG G		*Rsa I* restriction of A: AI: 473, 15 AII: 235, 202, 15	Amar et al, 2002
	TPIA nested	TPIA-IF: CCA AGA AGG CTA AGC GTG C TPIA-IR: GCC ACA TGC CTA TGT ACG GG	452		
	TPIB nested	TPIB-IF: GCA CAG AAC GTG TAT CTG G TPIB-IR: CTC TGC TCA TTG GTC TCG C	140		
gdh	1st round	GDHeF: TCA ACG TYA AYC GYG GYT TCC GT GDHiR: GTT RTC CTT GCA CAT CTC C	432	*Nla IV* restriction: AI: 150, 120, 90 AII: 120, 90, 80, 70 BIII&IV: 290, 120 C: 190, 120, 70 D: 250, 120 E: 220, 100, 80 *Rsa I* restriction of B: BIII: 300, 130 BIV: 430	Read et al, 2004
	2nd round	GDHiF: CAG TAC AAC TCY GCT CTC GG GDHiR: GTT RTC CTT GCA CAT CTC C			
β-giardin	1st round	G7: AAG CCC GAC GAC CTC ACC CGC AGT GC G759: GAG GCC GCC CTG GAT CTT CGA GAC GAC	511	Hae III restriction: A: 201, 150, 110, 50 B: 150, 117, 110, 84, 26 C: 194, 150, 102, 50 D: 200, 194, 117 E: 186, 150, 110, 26 F: 186, 150, 110, 50	Lalle et al, 2005
	2nd round	BGF: GAA CGA ACG AGA TCG AGG TCC G BGR: CTC GAC GAG CTT CGT GTT			

RESULTS

In order to determine the prevalent circulating giardial assemblages/genotypes in the region, stool samples from children with diarrhea, children with no diarrhea and adults with gastrointestinal symptoms that were microscopically positive for giardial cysts or trophozoites were included in this study.

Characteristics of study subjects

Children with giardial diarrhea: As mentioned, children with giardial diarrhea were enrolled from a community based birth cohort study on diarrhea. During the 3 year follow up of children in the birth cohort between March 2002 to August 2003, 155 episodes of giardial diarrhea were detected in 99 children, 53 of whom were male. The mean (SD) age at first episode was 1.6 (0.66) years. The characteristics of giardial diarrhea in this community have been described in Table 5. Although no child had persistent diarrhea, 1 child had diarrhea for 9 days and 5 children had diarrhea for 8 days. To identify risk factors associated with acquiring giardial diarrhea, socio-demographic data between 99 children, one or more episodes of giardial diarrhea was compared with the remaining 353 children in the birth cohort. Low socioeconomic status (OR, 95% CI) (1.62, 1.00 – 2.63) and drinking municipal water (1.69, 0.20 – 14.24) were associated with giardial diarrhea while sex, birth weight, maternal age, family size, number of siblings and presence of domestic animals in the household were not. Better maternal education (0.57, 0.33 – 1.01 for grades 1-5 and 0.31, 0.15 – 0.67 for grades 9 and above) and presence of a toilet at home (0.61, 0.35 – 1.08) were found to be protective. *Stool samples of 25 children from among these 99 were then randomly selected for the current study.*

Table 5: Characteristics of Giardial diarrhea among children in the community

Features of Giardial diarrhea	**N =155 episodes**
Acute (<4 days)	113 (72.9%)
Indeterminate (4 -14days)	42 (27.1%)
Persistent (> 14days)	0
Average duration of diarrhea (IQR)	2 days (2-4 days)
Maximum duration of diarrhea	9 days
	N= 99 cases
Multiple episodes	34 cases
Maximum number of episodes	5 episodes
Relapses	12 (7.7%)
Recurrences	44 (28.4%)
Median interval between recurrent episodes (IQR)	125 days (58 -167 days)
Presence of pets/domestic animals	14 (14.14%)

Children without diarrhea: Asymptomatic giardiasis was diagnosed among 64 children attending the urban health clinic. Twenty five asymptomatic controls were selected from among these children for the current study. The age of children without diarrhea included in this study ranged from 0.8 to 4.5 years, with the mean (SD) age of 2.6 (0.93). Among these children 13 (52%) were males and 12 (48%) were females.

Adults with gastrointestinal symptoms: Between September and October 2008, 58 samples received were positive for giardial cyst/trophozoites by microscopy among which 24 positive samples from patients above the age of 18 years were included in this study. The age of adult subjects ranged from 18 to 73 years, with a mean (SD) age of 41 (13.5) years. There were more male (66.7%) than female (33.3%) patients. Most of the adult subjects were from the state of West Bengal (66.7%) and only 2 cases were from Tamil Nadu.

The chart review indicated that abdominal pain (62.5%) was the most common symptom followed by bloating (45.8%), anorexia (33.3%), belching (25%) and nausea (25%). Weight loss, diarrhea, vomiting and fever were seen only in a few cases (Figure 3). Nearly half the charts (45.8%) also revealed other symptoms of functional bowel disease e.g.: passage of hard stool, feeling of incomplete evacuation, constipation etc. Clinical diagnosis mostly included dyspepsia and functional bowel disease including irritable bowel syndrome (Figure 4). Eight patients had other unrelated clinical diagnosis while 2 had no clinical diagnosis.

Fig 3 – Symptoms Associated with Giardiasis in Adult Patients (n=24)

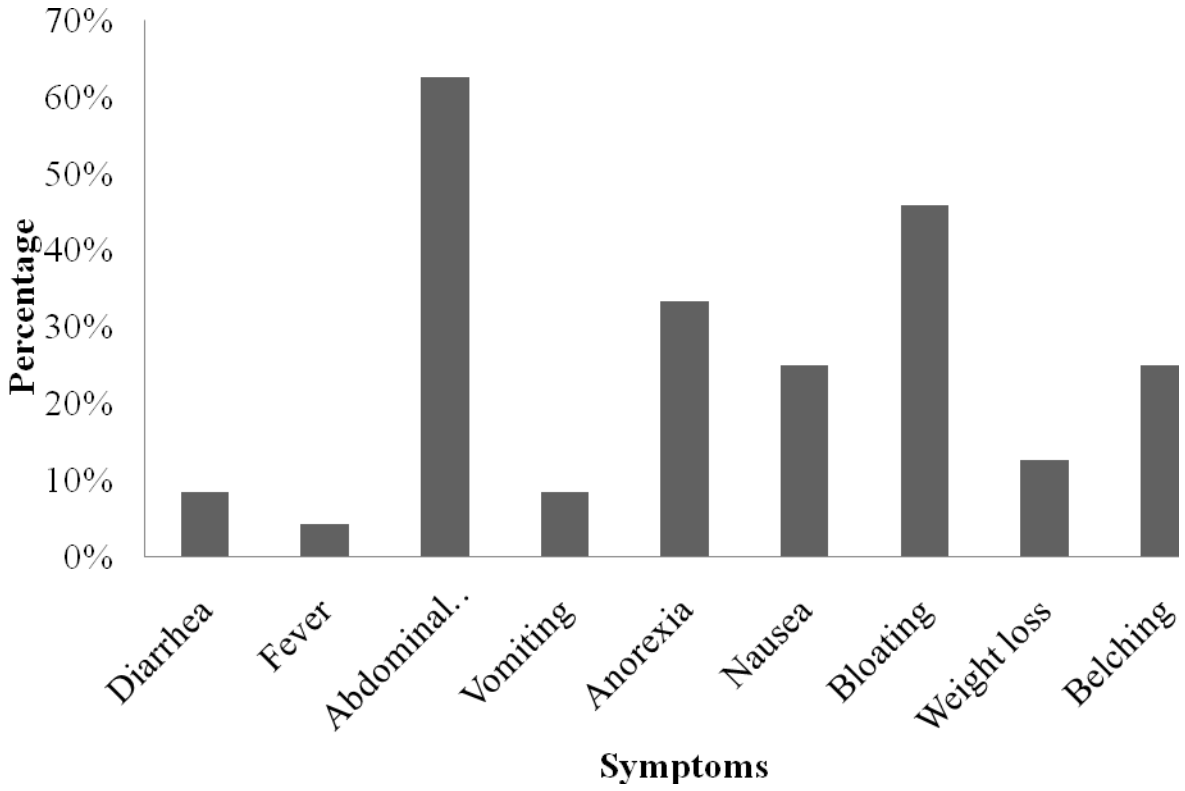
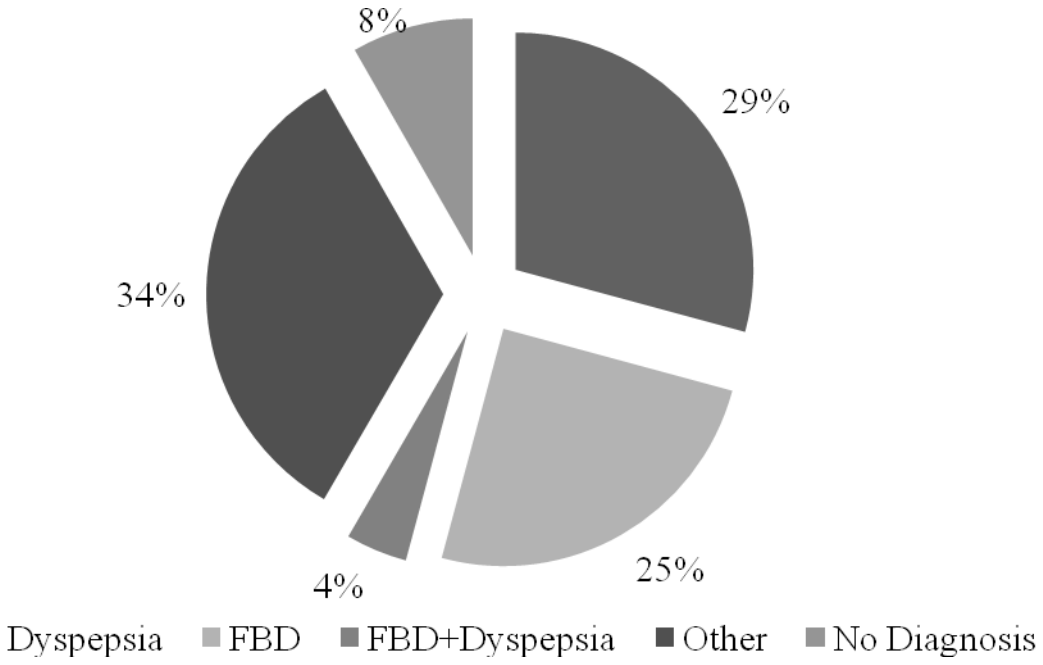


Figure 4 - Clinical Diagnosis in Adult Patients (n=24)



Validation of PCR RFLP results

For tpi PCR, assemblage AII and B were obtained by RFLP. Representative sequences of both these assemblages (CRI 9692 and CRI 8325, assemblage AII and CRI 2635, assemblage B) were sequenced and a BLAST search revealed >99% homology with sequences EU518551 (AII) and HM140723 (B), thus validating the RFLP results. For the gdh PCR, assemblages AII, BIII and BIV were obtained by RFLP. When the representative sequences (CRI 8325 and GS09, assemblages AII, AG26 and AG28 for assemblage BIII and AG19 for assemblage BIV) were analyzed by a BLAST search, not all results correlated with RFLP findings. While the CRI 8325 isolate was correctly identified as assemblage AII (>99% homology with EF507681), the AG26 and AG28 as assemblage BIII (>95% homology with GQ919299) and AG19 as assemblage BIV (>99% homology with EF507665), the GS09 sample was identified as assemblage AI. For the β-giardin PCR, assemblage A and B were identified by RFLP and when representative samples of assemblage A (AG23 and CRI 22079) and B (KB168 and GS16) were sequenced, a BLAST search revealed that the AG23 was assemblage AII (>99% homology with FN286482), the CRI 22079 was assemblage AIII (>99% homology with FN386484) and the 2 assemblage B isolates were BIII (>99% homology with DQ090528).

Assemblage distribution among samples

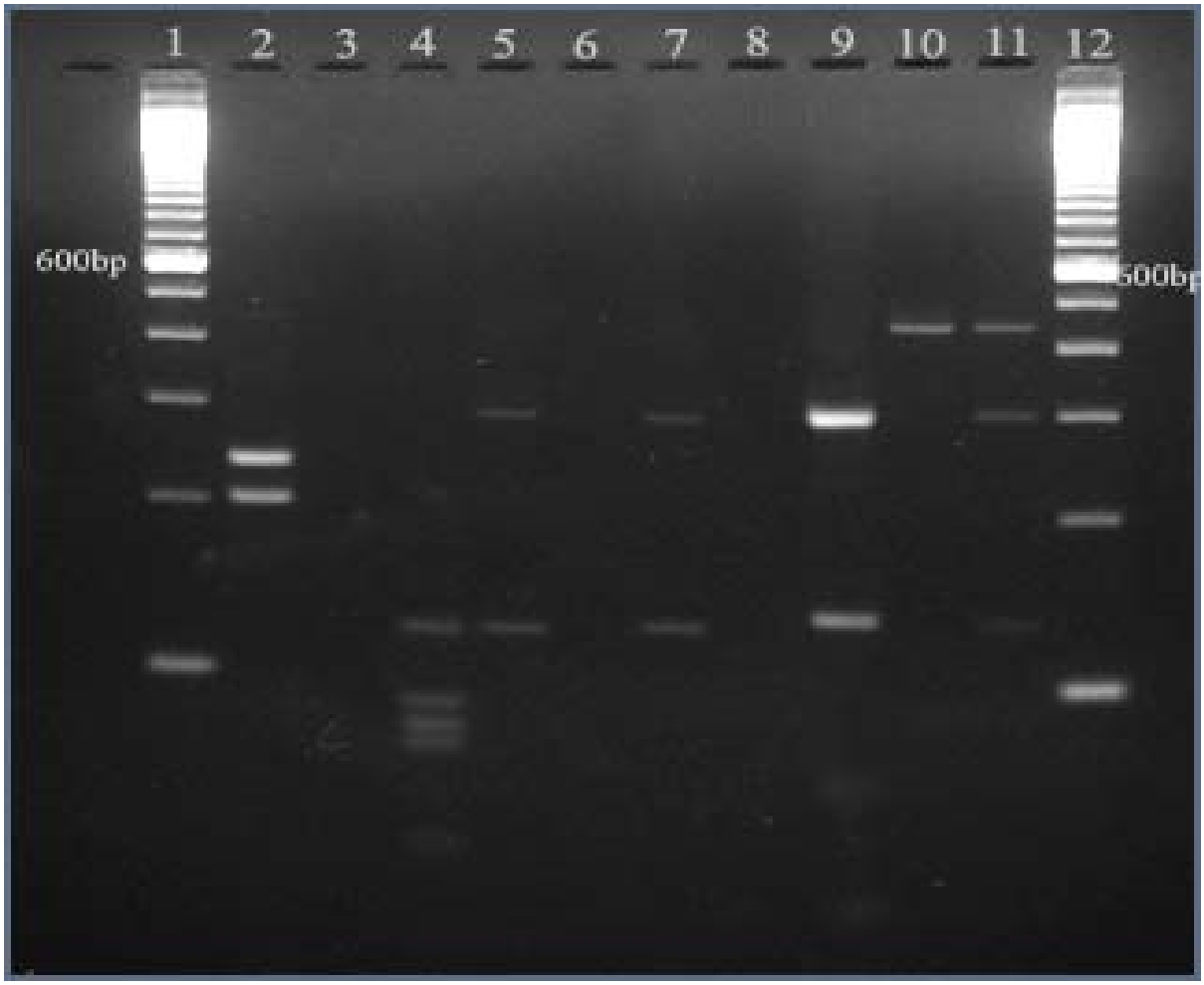
The tpi-PCR was positive for all samples included in this study (Figure 5 and 6). Among children with diarrhea 3/25 were found to have assemblage AII infection, 18 had assemblage B

and 4 were co-infected with assemblage A and B. Among children without diarrhea 2/25 were infected with assemblage AII, 21 with assemblage B and 2 had a co-infection with assemblage A and B. Among samples with an Assemblage A and B co-infection, the sub-group of the assemblage A could not be determined for 3 samples while the remaining 3 are subtyped as AII. Among the adult samples, 2/24 were infected with assemblage AII and 22 had an assemblage B infection. No co-infection was seen in adults. Assemblage AI infection was not detected in any of the samples by RFLP.

The gdh PCR-RFLP (Figure 5 and 6) was positive in 60% (15/25) of children with diarrhea. Among the positive samples, assemblage AII was detected in 3, BIII in 1 and BIV in 3 children. Co-infections with BIII and BIV were detected in 8 of these children. Among children without diarrhea, the gdh PCR was positive in 13 children (52%). Assemblage AII was detected in 1, BIII in 4 and BIV in 3 children without diarrhea. Additionally, 5 of these children had co-infections with BIII and BIV. Among the adult samples, more positives (18/24, 75%) were detected by gdh PCR than in samples from children. Among these samples, 1 assemblage AII infection, 8 BIII, 6 BIV infection and 3 BIII and BIV co-infections were detected. Assemblage BIII and BIV co-infection was seen more frequently in samples from children (13/28) than adults (3/18) (Fisher's exact, p=0.058). Similar to the tpi PCR-RFLP, no Assemblage AI was detected in any of the samples.

The β-giardin PCR (Figure 7) was positive in 56% (14/25) of children with diarrhea and among the positives, 3 assemblage A and 11 assemblage B samples were detected. Among children without diarrhea and adults, a similar number of samples was found to be positive

Fig. 5 - PCR RFLP at the tpi and gdh Loci to Determine Assemblage A and B



Lane 1 & 12: 100 bp molecular marker; lanes 3, 6 and 8: blank; lane 2: tpi-PCR -assemblage AII; lane 4: gdh-PCR -assemblage AII; lane 5, 7: gdh-PCR - assemblage B; lane 9: gdh-PCR BIII; lane 10: gdh-PCR assemblage BIV; lane 11: gdh-PCR co-infection with assemblage BIII & BIV

Figure 6 – Assemblage Distribution among Children with Diarrhea (n=25), Without Diarrhea (n=25) and Adults (n=24) for tpi and gdh loci

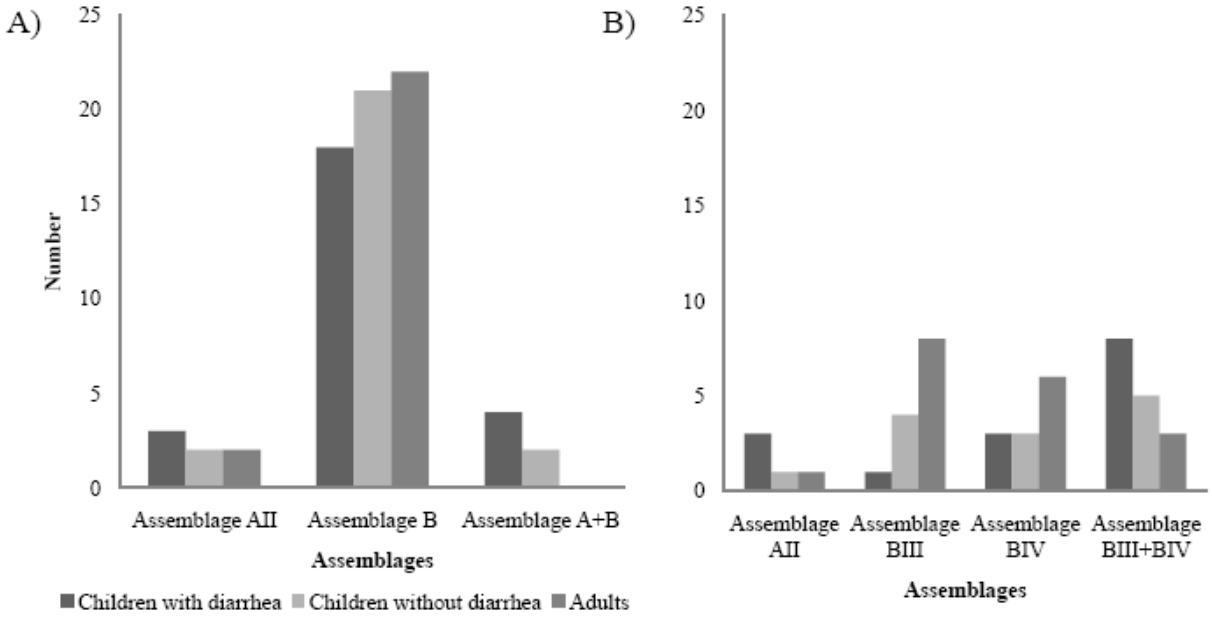
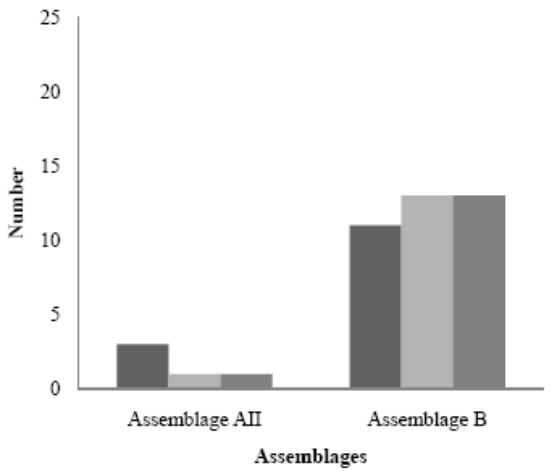


Figure 7 – β-giardin PCR A) RFLP to Determine Assemblage and B) Assemblage Distribution among Children with Diarrhea (n=25), Without Diarrhea (n=25) and Adults (n=24)

A)

B)



HaeIII restriction digestion Lane 1 & 4: 50bp molecular marker; lane 2: assemblage A lane 3: assemblage

(14/25 and 14/24 respectively). Among the positives, 1 assemblage A and 13 assemblage B were detected both in children without diarrhea and in adults. No co-infection was detected by the β-giardin PCR- RFLP in any group nor were any animal associated assembalges detected.

Comparison of genotyping methods

When the 3 genotyping methods are compared, tpi-PCR was found to be the most sensitive with amplification occurring in all of the samples. In comparison, the gdh-PCR and β-giardin PCR fared poorly with amplification in only 62.2% (46/74) and 56.8% (42/74) of the samples. The tpi-PCR was also able to resolve co-infections with assemblage A and B, while the other two methods could not. When samples that were positive for all 3 PCRs were compared (n = 37), those with an assemblage A and B co-infection (6 samples) detected by tpi-PCR-RFLP were typed as either A or B alone by the gdh and β-giardin PCR-RFLP (3/6 by gdh and 4/6 by β-giardin). These discordant results are given in Table 6. For single assemblage infections, no discordant results were seen between the 3 loci by RFLP. All the assemblage A detected were subtyped as AII by tpi and gdh-PCR-RFLP. No assemblage AI or any of the animal associated assemblages were detected by any of the 3 RFLP methods. However, sequencing of the few representative samples revealed 1 assemblage AI by gdh PCR that was typed as AII by RFLP at the tpi and gdh loci and 1 assembalge AIII by the β-giardin PCR that was typed as AII by RFLP at the tpi and gdh loci.

Table 6 : Genotyping results of the six samples with co-infection by tpi PCR-RFLP

Sample No.	Genotype(s) identified		
	tpi PCR-RFLP	gdh PCR-RFLP	β-giardin PCR-RFLP
KB 68	AII + B	Negative	Negative
KB 95	AII + B	Negative	Negative
CRI 11341	A + B	Negative	B
CRI 16468	A + B	BIII + BIV	B
CRI 20685	A + B	BIII	B
CRI 22079	AII + B	AII	A (AIII*)

*by sequencing

Association of symptoms with assemblage

When both single infection and co-infections were considered together, more children with diarrhea had assemblage AI infection (7/25) than children without diarrhea (4/25) but the association was not statistically significant probably due to the small sample size ($p = 0.496$). There was no difference in features of diarrhea like severity (measured as maximum number of stools per day), dehydration, duration or associated symptoms like vomiting and fever between children with assemblage A and B infection.

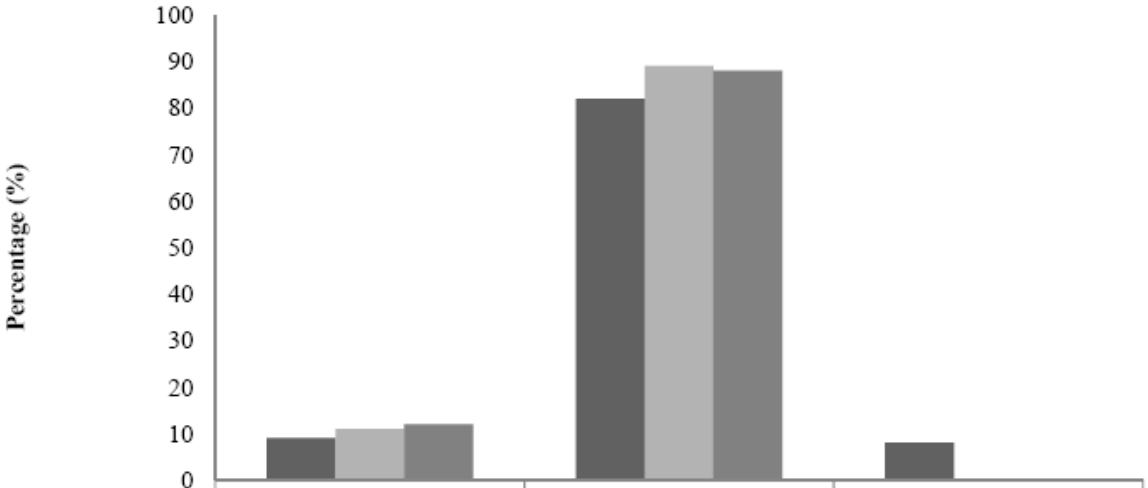
DISCUSSION

Giardiasis is a common enteric infection in India, but till date only three epidemiological studies with genotyping of isolates have been conducted (Paintlia, Descoteaux et al. 1998; Traub, Monis et al. 2004; Ajjampur, Sankaran et al. 2009). In order to better understand the molecular epidemiology of this ubiquitous organism, this study was conducted in three populations including children with diarrhea, children without diarrhea and adults complaining of gastro-intestinal symptoms. A MLG approach targeting three loci (tpi, gdh and β-giardin) was used for the characterization of the isolates.

In this study, assemblage B was found to be the predominant genotype among all the 3 groups studied and constituted 81.4% of the samples (Figure 8). Similar findings were obtained in a previous study from Vellore carried out on children with and without diarrhea (Ajjampur, Sankaran et al. 2009). Other studies from this region also described similar findings. These include studies from neighboring countries like Nepal and Bangladesh (Haque, Roy et al. 2005; Ng, Gilchrist et al. 2005; Singh, Janaki et al. 2009) and a previous study from north India (Paintlia, Descoteaux et al. 1998) and north-east India (Traub, Monis et al. 2004). Studies from south-east Asian countries like Thailand, Malaysia and Philippines also showed a higher prevalence rate of assemblage B than A (Yason and Rivera 2007; Mohammed Mahdy, Surin et al. 2009; Kosuwin, Putaporntip et al. 2010; Tungtrongchitr, Sookrung et al. 2010). The other

assemblage detected in this study was AII, which is also a predominantly human associated genotype. Assemblage AI, which is an animal associated assemblage (Sprong, Caccio et al.

Figure 8 - Assemblage distribution by different genotyping methods



	Assemblage A	Assemblage B	Co-infection A+B
tpi (N=74)	9	82	8
gdh (N=46)	11	89	0
β-giardin (N=42)	12	88	0

2009) was not detected in this study by RFLP, however, sequencing of a few representative samples did reveal the presence of both assemblage AI and AIII. Other potentially zoonotic animal associated assemblages (D – G) were not detected despite testing with the β-giardin and gdh PCRs which are able to differentiate several different assemblages.

The different methods used in the study also had differing resolutions. The tpi PCR-RFLP could resolve assemblage AI, AII and B; gdh PCR-RFLP resolved assemblage AI, AII, BIII, BIV and the animal associated assemblages C, D and E; and β-giardin PCR-RFLP resolved assemblage A, B and the animal assemblages C, D, E and F. The tpi PCR detected co-infection with assemblage A and B which were not detected by the gdh and β-giardin PCR-RFLP. Other studies have also reported co-infections using the tpi PCR that were not identified by PCRs at other loci (Geurden, Levecke et al. 2009; Lalle, Bruschi et al. 2009). Apart from the co-infections, in the remaining single infections there was no discordance among the results for the 3 PCRs by RFLP. However when representative isolates were sequenced, 2 isolates which had been typed as assemblage AII by the gdh and tpi PCR-RFLP were identified as assemblage AI and AIII. Although other studies have reported discordance between SSU rDNA, ef1-α and tpi-PCR and more so among animal samples (Read, Monis et al. 2004; Traub, Monis et al. 2004; Caccio, Beck et al. 2008), discordance between the RFLP and sequencing at the gdh loci has not been reported before (Read, Monis et al. 2004). These sequencing results imply that there is probably more heterogeneity in the samples tested than revealed by RFLP and the potential transmission of other zoonotic subtypes and assemblages needs to be studied further using sequencing.

When the sensitivity of the 3 PCR RFLP methods carried out were compared, the tpi PCR was the most sensitive for detecting and genotyping human associated assemblages, being positive in all samples. This high sensitivity (>90%) has also been found by other workers both to study sporadic cases and outbreaks using the same primers (Amar, Dear et al. 2002) as well as other primers targeting the tpi gene (Bertrand, Albertini et al. 2005; Tungtrongchitr, Sookrung et al. 2010). Studies that had lower sensitivity rates with the tpi PCR reported carrying out PCRs on formalin preserved stools (Molina, Polverino et al. 2007; Minvielle, Molina et al. 2008) while others had no possible explanation for the low sensitivity (Traub, Monis et al. 2004; Lalle, Bruschi et al. 2009).

In comparison to the tpi PCR, the gdh PCR had a poorer sensitivity with amplification in only 62.2% of samples. Similar findings have been observed for the gdh PCR (Bertrand, Albertini et al. 2005; Fallah, Nahavandi et al. 2008; Lalle, Bruschi et al. 2009; Tungtrongchitr, Sookrung et al. 2010). The performance of the gdh PCR was poorest in a study conducted on Peruvian children where the gdh PCR was positive in only 16 out of 210 microscopically positive samples (Perez Cordon, Cordova Paz Soldan et al. 2008). The β-giardin PCR also had a lower sensitivity with amplification in only 56.8% of samples. Lower rates of sensitivity have also been seen in other studies (Gelanew, Lalle et al. 2007; Lebbad, Ankarklev et al. 2008; Tungtrongchitr, Sookrung et al. 2010). Only a single study from Belgium found that β-giardin was more useful than tpi PCR (Geurden, Levecke et al. 2009).

A study that evaluated the analytical sensitivities of the SSU rRNA, β-giardin, gdh, tpi and ef1-α concluded that the SSU rRNA PCR was the most sensitive, possibly due to the high copy number of SSU rRNA in the organism, and due to its conserved nature (Nantavisai, Mungthin et al. 2007). Comparison of the tpi, gdh and β-giardin PCRs showed that the β-giardin PCR could amplify DNA at lower concentrations while the gdh PCR was more sensitive at lower cyst concentrations. However, although the sensitivities of the different methods seem to differ, different samples negative by one PCR may be detected by another PCR. In this study, only 37/74 samples were positive for all the three loci. Eight samples that were positive for gdh PCR were negative for β-giardin PCR, and 5 samples negative for gdh PCR were positive for β-giardin PCR. Similar findings have been documented by other workers when tpi and gdh PCR were compared (Lalle, Bruschi et al. 2009) and also when gdh or β-giardin PCR were used (Pelayo, Nunez et al. 2008). Thus, even with varying sensitivities, giardial genotypes are best determined by using a MLG approach.

Another interesting finding of this study was that co-infection with multiple giardial assemblages was seen predominantly in children. Assemblage A and B co-infection was seen exclusively in children (by tpi PCR) while co-infection with assemblage BIII and BIV was seen mainly in children (13/28 positive by gdh PCR) and detected in only 3 of 18 adults (p=0.058). The reason for this increased rate of multiple genotype infections in children is not clear. A study from Thailand and another from Ethiopia also reported a higher number of assemblage A and B co-infections among cases below the age of 15 years (Gelanew, Lalle et al. 2007; Tungtrongchitr, Sookrung et al. 2010). However, it is also important to note that the 'children' mentioned in this study were all at or under the age of 3 years when the sample was collected. In

another study in Brazil also carried out on a birth cohort of children under 3.5 years, mixed assemblage infections were always the first documented giardial infections and all subsequent re-infections were due to single infection (Kohli, Bushen et al. 2008). Several population based serological studies have also found increasing rates of seropositivity with age indicating exposure and possibly development of immunity to giardiasis (Cedillo-Rivera, Leal et al. 2009). This could potentially explain the increased rates of co-infections among younger age groups. Contrary to these findings, Yason et al, found co-infection to be more frequent in older age groups (Yason and Rivera 2007). However the number of people in the > 15 year age group in the study was very small (n=6).

In this study, although there were more children with diarrhea who had assemblage A infection, the association with diarrhea was not significant. However, in a previous study from Vellore on children with and without diarrhea with a larger number of samples, assemblage AII single infection or co-infection with assemblage B was associated with diarrhea ($P = 0.07$). Interestingly, most previous studies (6 of 8) that found an association between assemblage A and the occurrence of diarrhea or other gastrointestinal symptoms also recorded a higher prevalence rate of assemblage B (Figure 2). The studies with a predominance of assemblage B were from Australia, Turkey, Bangladesh, Spain, England and India (Vellore) (Read, Walters et al. 2002; Aydin, Besirbellioglu et al. 2004; Haque, Roy et al. 2005; Sahagun, Clavel et al. 2008; Ajjampur, Sankaran et al. 2009; Breathnach, McHugh et al. 2010) while the 2 studies that had both predominance of and association of symptoms with assemblage A were from Peru and Egypt (Perez Cordon, Cordova Paz Soldan et al. 2008; Helmy, Abdel-Fattah et al. 2009). However, in some studies a correlation of symptomatic or severe infection was found with assemblage B.

The predominant circulating assemblage was assemblage B in 3 studies (Netherlands, Malaysia and Thailand) (Homan and Mank 2001; Mohammed Mahdy, Surin et al. 2009; Tungtrongchitr, Sookrung et al. 2010) and assemblage A in 1 study (Ethiopia)(Gelanew, Lalle et al. 2007) while a study from Cuba had equal distribution of assemblage A and B (Pelayo, Nunez et al. 2008). This trend, in which in most studies the predominant circulating genotype causes asymptomatic infections while the minor genotype causes symptomatic infection, may be explained by host adaptation or immunity to the predominant genotype (Haque, Roy et al. 2005) but the conflicting data suggests that there are probably other host, parasite and epidemiological factors involved.

The main limitations of the study were the small sample size and the lack of a longitudinal follow up. A study with larger number of samples with homogenous socio-demographic profile may help better understand the relationship between infecting genotype and the development of symptoms. The occurrence of multiple genotypes in children can also be better studied in a longitudinal study. The MLG tool used was PCR RFLP and may have had a poor resolution to detect discordance between the loci or multiple infecting genotypes (Caccio, Beck et al. 2008; Geurden, Levecke et al. 2009; Tungtrongchitr, Sookrung et al. 2010).

In conclusion, assemblage B was the predominant circulating assemblage both in adults and children in India and children were more prone to develop co-infections with multiple assemblages or subgenotypes. These findings suggest that giardiasis in this region is mostly associated with an anthroponotic transmission cycle, but to identify zoonotic transmission future studies with a more sensitive tool like sequencing need to be carried out.

SUMMARY

- Assemblage B was the predominant genotype in both adults and children (with and without diarrhea) in India with assemblage AII being the second most common.
- Tpi-PCR RFLP was the most sensitive among the 3 genotyping methods used.
- Co-infections with multiple assemblages and subgenotypes were seen more commonly in children. Assemblage A and B co-infections were seen exclusively and BIII and BIV more frequently in children.
- Some discordance of results was seen between the 3 methods used especially when sequencing was carried out warranting future more detailed studies.
- Though not statistically significant, more children with diarrhea had assemblage A infection either as single infection or as co-infections with assemblage B.

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APPENDIX I
Approval letter of the Institutional Review Board, Christian Medical College, Vellore

CHRISTIAN MEDICAL COLLEGE
VELLORE - 632 002, INDIA.
INSTITUTIONAL REVIEW BOARD (IRB)

Dr. George Thomas, D.Orth
Editor Indian Journal of Medical Ethics
Chairman, Ethics Committee

Dr. Asha Mary Jesudasan, MD,PhD
Chairman, Research Committee &
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Dr. Shuba Kumar, PhD
Deputy Chairman, Ethics Committee

Dr. Gagandeep Kang, MD,PhD,FRCPath
Deputy Chairman, IRB &
Additional Vice Principal (Research)

Dr. L. Jeyaseelan, MSc,PhD
Secretary, IRB

February 2, 2009

Dr. Shakti Laishram
Clinical Microbiology
Christian Medical College
Vellore – 632 004.

Sub: FLUID Research grant project NEW PROPOSAL:
Multi locus genotyping of giardial assemblages associated with diarrhea in children and adults in South India.
Dr. Shakti Laishram, PG Demonstrator, Clinical Microbiology, Dr. Gagandeep Kang, Dr. Sitara Swarna Rao Ajjampur, Mr. Arun Kannan, GI Sciences.

Ref: IRB Min. No. 6684 dated 22.10.2008.

Dear Dr. Laishram,

The Institutional Review Board (Ethics Committee) of the Christian Medical College, Vellore, reviewed and discussed your project entitled "Multi locus genotyping of giardial assemblages associated with diarrhea in children and adults in South India" on October 22, 2008.

The Committees reviewed the following documents:

1. Format for application to IRB submission.CVs of Drs. Shakti Laishram, Gagandeep Kang, Sitara Swarna Rao Ajjampur, Arun Kannan.
2. A CD containing documents 1-2.

The following Ethics Committee members were present at the meeting held on October 22, 2008 at 10:00 am in the CREST/SACN Conference Room, Christian Medical College, Bagayam, Vellore 632002.

Name	Qualification	Designation	Other Affiliation
Dr. George Thomas	MBBS, D.Ortho	Chairperson (IRB) & Orthopaedic Surgeon, St. Isabel Hospital, Chennai & Editor, Indian Journal of Medical Ethics	Non-CMC Staff.
Dr. Thambu David (on behalf of Dr. Lionel Gnanaraj)	MBBS, MS, M.Ch. (Urol)	Medical Superintendent, CMC.	

TEL : 0416 - 2284294, 2284202 FAX : 0416 - 2262788
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CHRISTIAN MEDICAL COLLEGE
VELLORE - 632 002, INDIA.
INSTITUTIONAL REVIEW BOARD (IRB)

Dr. George Thomas, D.Orth
Editor Indian Journal of Medical Ethics
Chairman, Ethics Committee

Dr. Asha Mary Jesudasan, MD,PhD
Chairman, Research Committee &
Principal

Dr. Shuba Kumar, PhD
Deputy Chairman, Ethics Committee

Dr. Gagandeep Kang, MD,PhD,FRCPath
Deputy Chairman, IRB &
Additional Vice Principal (Research)

Dr. L. Jeyaseelan, MSc,PhD
Secretary, IRB

Mrs. Jasmine Anand (on behalf of Mrs. Sundari Edwin)	M.Sc. (Nursing)	Nursing Superintendent, CMC.	
Rev. Dr. T. Arul Dhas	M.Sc., BD, Ph.D.	Chaplain, CMC	
Mr. Harikrishnan	BL.	Lawyer	Non-CMC staff.
Dr. Denise Fleming	B.Sc., Ph.D.	Professor, Pharmacology Dept. CMC.	
Dr. Srinivas Babu	MSc, Ph.D.	Sr. Scientist, Neurological Sciences, CMC.	
Dr. Varghese Cherian	MBBS, DA, MD.	Professor, Anaesthesia Dept. CMC.	
Mrs. S. Pattabhiraman	BSc, DSSA	Social Worker, Vellore	Non-CMC-Staff
Dr. Gagandeep Kang	MD, PhD, FRCPath.	Dy. Chairperson (IRB), Professor of Microbiology & Addl. Vice Principal (Research), CMC.	

We approve the project to be conducted in its presented form.

The Institutional Ethics Committee / Independent Ethics Committee expects to be informed about the progress of the project, any SAE occurring in the course of the project, any changes in the protocol and patient information/informed consent and asks to be provided a copy of the final report.

A sum of Rs. 60,000/- (Rupees sixty thousand only) is sanctioned for 2 years and out of which a maximum of Rs 1,500/- can be spent for stationery, printing, Xeroxing and computer charges (if computers used are within the institution).

Yours sincerely,

L. Jeyaseelan.

Dr. L. Jeyaseelan, PhD
Secretary, IRB

Secretary
Institutional Review Board
(Ethics Committee)
Christian Medical College
Vellore - 632 002, Tamil Nadu, India

TEL : 0416 - 2284294, 2284202 FAX : 0416 - 2262788
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APPENDIX II

Genotyping results of the samples by the three different methods

Group	Study No.	tpi	gdh	β-giard
1	AG1	B	0	0
1	AG2	B	BIV	B
1	AG3	B	BIV	B
1	AG4	B	BIV	B
1	AG6	B	BIV	B
1	AG7	B	0	0
1	AG9	B	BIII	0
1	AG11	AII	0	0
1	AG12	B	BIII+IV	B
1	AG13	B	0	0
1	AG14	B	BIII	B
1	AG15	B	BIV	B
1	AG17	B	0	0
1	AG18	B	BIII	0
1	AG19	B	BIV	B
1	AG21	B	BIII	B
1	AG23	AII	AII	A
1	AG25	B	0	0
1	AG26	B	BIII	B
1	AG27	B	BIII	0
1	AG28	B	BIII	B
1	AG29	B	BIII	0
1	AG31	B	BIII+IV	B
1	AG36	B	BIII+IV	B
2	CRI 4278	B	0	0
2	CRI 8178	AII	0	0
2	CRI 8325	AII	AII	A
2	CRI 8449	B	BIV	0
2	CRI 11277	B	BIII+IV	B
2	CRI 11341	A+B	0	B
2	CRI 11343	B	0	0
2	CRI 12635	B	BIV	B
2	CRI 13508	B	BIII+IV	B
2	CRI 13682	B	0	0
2	CRI 13992	B	0	0
2	CRI 14619	B	BIV	0
2	CRI 16468	A+B	BIII+IV	B

Group	Study No.	tpi	gdh	β-giard
2	CRI 17740	B	0	0
2	CRI 20544	B	BIII+IV	B
2	CRI 20685	A+B	BIII	B
2	CRI 22079	A+B	AII	A
2	CRI 22131	B	0	0
2	CRI 22212	B	BIII+IV	B
2	CRI 24084	B	BIII+IV	B
2	CRI 24152	B	BIII+IV	B
2	CRI 24918	B	0	0
2	CRI 25153	B	BIII+IV	B
2	CRI 25445	B	0	0
2	CRI 25888	AII	AII	A
3	CRI 36591	B	0	0
3	CRI 36592	B	BIII+IV	B
3	CRI 39378	B	BIII+IV	B
3	KB2	B	BIII+IV	B
3	KB35	B	BIII	B
3	KB61	B	0	B
3	KB68	A+B	0	0
3	KB76	B	BIII	B
3	KB94	B	BIII+IV	B
3	KB95	A+B	0	0
3	KB168	B	0	B
3	KB193	AII	0	A
3	KB36	B	BIII	0
3	KB141	B	0	0
3	KB162	B	0	0
3	KB7	B	0	0
3	GS09	AII	AII	0
3	GS16	B	BIV	B
3	GS19	B	BIV	B
3	GS22	B	BIV	0
3	GS33	B	BIII	B
3	KB62	B	0	0
3	KB127	B	0	0
3	KB130	B	0	B
3	KB136	B	BIII+IV	B

APPENDIX III

Symptomatology of the adult subjects

Study No.	Diarrhea	Freq	Fever	Pain abd.	Vomiting	Anorex.	Nausea	Bloating	Fullness	Wt loss	Belching	Others/s	Clin. Diag.
AG1	0	0	0	1	0	0	0	1	0	0	1	0	IBS
AG2	0	0	0	1	0	1	0	1	0	0	1	1	Dyspepsia
AG3	0	0	0	1	1	1	1	0	0	0	1	1	Others
AG4	0	0	0	1	0	0	0	1	0	0	0	1	FBD
AG6	0	0	0	1	0	0	0	0	0	0	0	1	Others
AG7	0	0	0	1	0	0	1	0	0	0	0	0	Others
AG9	1	12/d	1	1	0	1	1	0	0	1	0	0	Others
AG11	0	0	0	0	0	0	0	0	0	0	0	0	Others
AG12	0	0	0	1	0	1	0	0	0	1	1	1	IBS
AG13	0	0	0	1	0	1	0	0	0	0	1	0	Dyspepsia
AG14	0	0	0	0	0	0	1	1	0	0	0	1	Dyspepsia
AG15	0	0	0	1	0	0	0	0	0	0	0	1	Others
AG17	0	0	0	1	0	1	1	1	0	0	0	1	Dyspepsia
AG18	1	5/d	0	1	0	0	0	1	0	0	0	0	IBS
AG19	0	0	0	0	1	1	1	0	0	0	0	0	Others
AG21	0	0	0	0	0	0	0	0	0	0	0	1	IBS
AG23	0	0	0	0	0	0	0	0	0	0	0	0	Others
AG25	0	0	0	0	0	0	0	1	0	0	0	0	Dyspepsia
AG26	0	0	0	0	0	0	0	1	0	0	0	1	0
AG27	0	0	0	0	0	0	0	0	0	0	0	0	0
AG28	0	0	0	1	0	0	0	1	0	1	0	1	Dyspepsia+ FBD
AG29	0	0	0	0	0	0	0	0	0	0	0	0	FBD
AG31	0	0	0	1	0	0	0	1	0	0	0	0	Dyspepsia
AG36	0	0	0	1	0	1	0	1	0	0	1	0	Dyspepsia